Somatic embryogenesis and plant regeneration in cassava (Manihot esculenta, Crantz)

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SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN CASSAVA

(*Manihot esculenta ,Crantz)*

submitted by

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To my parents, sister, brothers, my husband Kaharuddin and my son Reza

ABSTRACT

Leaf lobes explants from young leaves (1-3 and 3-5 mm) of three South American and 15 African cassava cultivars were able to undergo somatic embryogenesis at a frequency varying between 10% and 100% on semi-solid MS medium containing 2% sucrose and 4.0 mgl^{-1} 2,4-D. Explants from two other South American cultivars failed to produce somatic embryos on this medium, but they were able to undergo embryogenesis on medium supplemented with either picloram (6.0-12.0 mgl^{-1}) or dicamba (10.0-66.0 mgl^{-1}). The maturity of explants, the concentration of 2,4-D, picloram and dicamba, and the period of incubation on Stage-I medium were shown to be critical factors to obtain somatic embryos at a high frequency.

Continuous production of somatic embryos of cassava cultivar CMC 76, which provided the most responsive explants, was established by subculturing clumps of somatic embryos and embryogenic tissues every month for three or four passages on semi-solid MS medium supplemented with 4.0 mgl^{-1} 2,4-D and one passage on medium supplemented with 2.0 mgl^{-1} 2,4-D. It was important to discard the non-embryogenic callus and mature somatic embryos possessing green cotyledons at every subculture.

Somatic embryos were shown to be a good source of cassava protoplasts whose development was affected by the concentration of 2,4-D. Both 2,4-D and NAA in combination with BAP in culture medium were shown to be essential for protoplast development.

Embryogenic suspension cultures of cassava were established when embryogenic tissues were cultured in liquid MS medium with 2,4-D at an initial concentration of 4.0 mgl^{-1}; the shaking rate and the size of the initial inoculum were shown to be critical. Embryogenic clumps obtained from the suspension cultures underwent secondary embryogenesis when they were plated on MS semi-solid medium supplemented with auxin, in particular 2.0 mgl^{-1} 2,4-D and regenerated to mature embryos following transfer to hormone-free MS medium.

A high frequency of normal plantlets could be regenerated from somatic embryos if an appropriate size (1-3 mm) of individual somatic embryos were cultured on hormone-free MS medium. Both somatic embryos and plantlets had a chromosome number 2n = 36.

A successful protocol for the cryopreservation of embryogenic tissues and somatic embryos of cassava was established. 25% of frozen somatic embryos produced secondary embryos and 66.7% of frozen somatic embryos produced callus were obtained with 1.5-2.0 mm somatic embryos cultured for four days before freezing on medium supplemented with 0.05 mgl^{-1} 2,4-D and 7% sucrose, and slow cooled (0.3^{o}C min^{-1}) to -30^{o}C prior to immersion in liquid nitrogen. After immersion in liquid nitrogen for at least 15 minutes, frozen tissues were thawed and washed in liquid medium at 22^{o}C prior to culture on the regeneration medium of the same composition as that used for culturing tissues before freezing. Plants regenerated both from embryogenic tissues and somatic embryos were apparently morphologically normal.

CONTENTS

ABBREVIATIONS

2,4-D, D	2,4-Dichlorophenoxyacetic acid
2n	diploid
ABA	Abscisic acid
BAP	6-Benzylaminopurine
B5	B5 medium, see Gamborg *et al* (1968)
casein hydr.	Casein hydrolysate
CIAT	Centro International de Agricultura Tropical
	Cali, Columbia
d	days
Dicamba	3,6-dichloro-o-anisic acid
DMSO	Dimethylsulfoxide
FAA	Formalin/acetic acid/ethanol
hr	hours
IAA	Indole-3-acetic acid (3-Indolyacetic acid)
IBA	Indole butyric acid
IITA	International Institute of Tropical Agriculture
	Ibadan, Nigeria
KIN, K	Kinetin (6-furfurylaminopurine)
L. compost	Levington compost
MS	Murashige and Skoog basal medium (1962)
NAA	Napthalene-acetic acid (1-Napthaleneacetic acid)
PAR	Photosynthetically Active Radiation
rpm	revolutions per minute
TBA	Tertiary butyl alcohol
Zeatin, Z	(6-[4-Hydroxy-3-methylbut-2-enylamino] purine)

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CHAPTER 1

GENERAL INTRODUCTION

1.1. CASSAVA

1.1.1. Origin, Description and Improvement of Cassava

Cassava (*Manihot esculenta*, Crantz) is a dicotyledonous perennial shrubby plant of the family Euphorbiaceae. Originating in North East Brazil, and probably Central America, it has been cultivated there for several thousand years following domestication (Cobley, 1977; Coursey and Booth, 1977; Odigboh, 1983; Cock, 1985). From the Brazilian Amazon region, cassava spread to the West Indies, Southeast Asia, and later to Africa in the sixteenth and seventeenth centuries (Jones, 1959; Jennings, 1976).

The cassava plant, which is monoecious and cross-pollinated by insects, flowers and produces fruits regularly in some cultivars (Onwuewe, 1978; Odigboh, 1983). Cassava seeds have been reported to be recalcitrant because after six months storage at laboratory temperature and between 5.9 and 1.9 percent moisture content (fresh weight basis) germination was reduced from 80 to 28 percent (Mumford and Grout, 1978).

Cassava, which has a chromosome number 2n=36, is a deciduous plant that grows to a height of 1 to 3 m or more depending on the variety and growing conditions. Its branching pattern is a varietal characteristic; there may be several main stems which branch at one-third, halfway, or two-thirds of the main stem or at the apex of the stem. There are also varieties which do not branch (Onwuewe, 1978; Odigboh, 1983). The leaves vary in shape, colour, size, in degree of dissection and width of the lobes; the petioles vary in length, colour and hairiness, and the stipules in colour and size. The leaves are usually dark green, but red, yellow and various shades of purple pigmentation occur in the foliage (Cobley, 1976). CIAT researchers recently discovered that in

cassava, the only known crop species which is an intermediate C_3/C_4 plant, there is a direct relationship between the photosynthetic capacity of the leaves and root yield (CIAT report, 1988).

Like branching pattern, the distribution of roots and tubers in a plant is a varietal characteristic; however, it can be influenced by the orientation in which the original stem cutting was planted (Onwuewe, 1977). Cassava tubers, which are swollen lateral roots, are usually cylindrical and unbranched (Acland, 1971).

1.1.2. The Importance of Cassava in The World and in Indonesia

As a tropical root crop, cassava is an important major source of calories to nearly 500 million people throughout the world (Rogers, 1965; Jennings, 1976). It is considered as a primary source of carbohydrate with a yield much higher than that of maize or rice and second only to yam (de Vries *et.al.*, 1967; Kawano *et.al.*, 1978; Odigboh, 1983). Caneiro and the Castro (1988) stated that the world consumption per person of tropical root crops is 57 kg/year and about a half of the amount is related to cassava.

According to F.A.O. (1986), cassava is one of the major food crops in the world with a total annual production of over 100 million metric tons. The world's chief producer is still Brazil with more than 25 million metric tons per year, followed by Zaire, Thailand, Nigeria and Indonesia (Table 1.1).

Table 1.1. World's Major Cassava Producer

Country	Area harvested (1000 ha)	Production (1000 m tons)
Brazil	2049	25542
Zaire	2200	15570
Thailand	1704	15255
Nigeria	1300	14700
Indonesia	1214	13329

FAO Production Year Book, 1986.

In Indonesia, where the storage roots of cassava are widely used for human consumption, cassava is the third most important food energy crop after rice and maize (Effendi, 1979). Studies in Indonesia, as one of the world's major cassava producers, have shown that per capita purchases of fresh cassava roots tend to increase as the income level increases and they then remain stable at higher income levels (Cock, 1985). Due to the fact that Indonesia could produce 8% of the world's starch, several industrial plants have been built to produce fructose syrup in order to replace imports of sugar (Cooke and Cock, 1989). The area planted to cassava in Indonesia such as in the island of Sumatra, has responded to the situation of demand being greater than supply. Therefore in order to improve the nutrition of the lower income groups, to reduce dependence on imported grains and even to moderate demand for imported oil by

serving as an energy source, Indonesia has expanded cassava production (Cock and Lynam, 1982).

Cooke and Cock (1989) stated that people in the world eat more than 60% of cassava produced; about a third of the harvest feeds animals and the rest is transformed into secondary products such as starch, which is the major organic component of the cassava tuber (68.4% of the dry weight). Oyenuga (1968) and Blanshard (1988) believe that besides starch, the tuber is relatively rich in vitamin C (35 mg per 100 g fresh weight); there are also traces of niacin and vitamins A, B_1, and B_2. The tuber also contains relatively small amounts of protein (1-2%) which is rich in arginine, but low in methionine, lysine, tryptophan, phenylalanine and tyrosin (Oyenuga, 1968). In addition to the constituents already described, the tuber, contains small but significant amounts of cyanogenic glucosides (linamarin and lotaustralin are the two major components). The amount of these components, is however affected by cultivar, environment such as soil conditions, and the age of the plant (Onwuewe, 1978).

Since starch and sugar are the predominant components of the dry matter of the root, cassava is an important raw material in many industries such as sand paper, textiles, cosmetics and livestock feeds (Jennings, 1976; Odigboh, 1983; Cock, 1985; Biggs *et.al.*, 1986). Cooke and Cock (1989) argue that the future of cassava may lie with its secondary products : cassava chips make a nutritious animal feed; ground cassava can partially substitute for wheat in bread or it can be used with 50% rice flour to make low cost noodles; and cassava can be fermented to make alcohol and single-cell protein. Starch and starch derivatives, such as dextrins, glucose and high-fructose syrup are the main product of the cassava agroindustry. Currently, the importance of cassava as an energy source is manifested by its growing demand in the European Economic Community countries as well as other developed countries where cassava is

valued as a cereal substitute, competing favourably in prices for livestock feeds. Also, Brazil has been pursueing a national programme to replace by 1990 all petroleum consumption with alcohol distilled principally from cassava.

Unlike many other major world crops (rice, maize, etc.), cassava has not been subjected to extensive improvement programmes, although it has been shown that considerably improved yields of more than 60 tonnes per hectare can be achieved through breeding and selection (CIAT Report, 1982).

Onwuewe (1978) lists eight main areas for cassava improvement : (1) resistance to pest and diseases, (2) yield, (3) starch and low root-fibre content, (4) reduced cyanogenic glucoside content, (5) varieties which mature early but which do not deteriorate immediately on harvest, (6) high protein content, (7) stout, non-spreading roots and minimum of foliage, and (8) adaption to a wide-range of environmental conditions. By utilising existing variation, hybridisation and selection have contributed to the improvement of cassava. Apart from simple selection techniques, cassava improvement depends largely on traditional breeding techniques incorporating long-term hybridisation and selection programmes.

The regularity in flowering and setting seeds, and also the difference in terms of time and space of the male and female flowers are important factors in cassava improvement research. Mutation breeding with cassava has taken the form of treating the seed, seedling, or young shoots with mutagenic agents such as X-rays and -rays. The production of haploid plants as a crop improvement measure in cassava is a recent innovation that has not yet been completely developed (Moh, 1975).

1.1.3. Propagation of Cassava

1.1.3.1. Traditional propagation of cassava

As cassava is a highly heterozygous plant, it is traditionally propagated vegetatively by stem cuttings. A mature cassava plant will give between 10 and 30 normal sized (25 cm) stem cuttings for planting after one year (Holey and Lozano, 1976; Cock, 1985). It seems that the number of nodes on a cutting may be just as important as the absolute length of the cuttings. The part of the stem from which the cutting is taken influences the yield that might be expected from it (Onwuewe, 1978; Odigboh, 1983), with the longer cutting producing the greater the yield. However, practical considerations have dictated the use of shorter cuttings in many cassava-growing regions.

In addition to length of cuttings and the number of nodes on the cuttings, the tuber yield is also affected by the age of the cuttings and the orientation of cuttings when they are planted in the field. Depending on the variety, following propagation by stem cuttings, cassava tubers can be harvested 6-15 months after planting (Onwuewe, 1978; Odigboh, 1983). Propagation by seed is slow and uncertain, and moreover plants derived from seeds are smaller and weaker (Onwuewe, 1978).

1.1.3.2. In vitro propragation of cassava

Rapid methods of multiplication are necessary to satisfy the high demand for cassava which is increasing in parallel with the increase of the world's population and

the development of technology. It is predicted that the world's population will double in 50 years, and that most of this increase will occur in developing countries, which are concentrated in the tropics (Cock, 1985). Increased cassava production, can only be obtained if good technology is available for the efficient production of the crops. New techniques are, therefore, required to solve problems in propagating cassava, particularly those related to virus diseases which causes a considerable losses in cassava yield. It is possible that breeding for resistance to virus diseases, especially mosaic, and to bacterial blight disease, for high yielding varieties, high methionine/protein content varieties and for other desirable characters could be achieved more easily by *in vitro* methods alone or in combination with genetic engineering techniques.

Before *in vitro* techniques can be employed for plant improvement, however, it is essential that reliable methods should be developed for the regeneration of plants from callus and cell cultures. This applies equally when such techniques are used as an integral part of genetic engineering procedures. There have been reports of shoot regeneration from stem callus cultures of cassava (Tilquin, 1979) and from protoplasts (Shahin and Shepard, 1980), but these results seems to have been genotype-specific and the most promising method seems to be based on somatic embryogenesis in cultures derived from cotyledons and immature leaf lobes (Stamp and Henshaw, 1983). When the present work commenced, it was already apparent that the latter method was very much more reliable than the previously reported approaches, but it was still necessary to establish its usefulness with a wide range of genotypes, to devise procedures for the large-scale production of somatic embryos and to demostrate whether embryogenic competence can be maintained in cultures derived from single cells or protoplasts.

1.2. SOMATIC EMBRYOGENESIS

1.2.1. The nature of somatic embryogenesis

Somatic embryogenesis is one of the key *in vitro* techniques, with particular relevance to plant breeding and has potential for rapid propagation. Bell (1965) stated that the phenomenon of embryogenesis is not necessarily confined to the reproductive cycle and that it could be fairly inferred from the recent spectacular developments in the culture of single cells of higher plants that any diploid cell, in which irreversible differentiation has not proceeded too far, would, if placed in an appropriate medium, develop in an embryo-like way and produce a complete plant. In practice, it is difficult to decide when irreversible differentiation has proceeded too far or whether an appropriate medium has been employed, but it is now clear that in some species, including cassava (Stamp and Henshaw, 1984) embryogenic competence appears to be highly localized; moreover, the loss of competence is not usually associated with visible signs of irreversible differentiation. At present, therefore, it is not possible to say whether the loss of embryogenic competence in some members of a population of otherwise in distinguishable cells, is a consequence of irreversible changes in the properties of those particular cells.

It is believed that in general the organizational events in somatic embryogenesis are similar to those of zygotic embryogenesis. Nevertheless, it has been shown that there are differences between somatic embryos and zygotic embryos. Firstly, one feature of somatic embryos is the continuation of cell divisions resulting in embryos larger than their zygotic counterparts. When the cotyledons begin to grow, they form a broad

disk-shaped meristem with many cell divisions visible around the periphery and in the centre. The cotyledons often appear fused along their margins producing a fasciated, tube-like structure rather than separate, distinct cotyledons. Secondly, there is premature cell enlargement and differentiation of somatic embryos. In carrot, during normal zygotic embryogenesis the cells remain small, densely cytoplasmic and apart from some changes in shape, undifferentiated. In somatic embryos, highly vacuolated cells are clearly visible by the absence of intense Feulgen-staining, especially in the hypocotyl region. Therefore, in somatic embryo development extended cell division in enlarged cells, altered spindle orientations, early cell differentiation and delayed initiation of cotyledons and shoot apex can occur (Ammirato, 1987).

There might also be differences between zygotic and somatic embryos, in terms of their origin. Haccius (1978) defines that both zygotic and somatic embryos are new individuals arising from single cells and having no vascular connection with maternal tissues. However, work with immature zygotic embryos of *Trifolium repens* showed that multicellular budding and single-cell initiation apparently both occur, with multicellular budding being the more frequent pattern (Maheswaran and Williams, 1985). It seems that both direct or indirect somatic embryogenesis apparently may also occur by either one or both of these patterns. The occurence of direct somatic embryogenesis by multiple-cell budding has been discussed by James *et.al.* (1984); Button *et.al.* (1974) and Tisserat *et.al.* (1975) both suggested that somatic embryos develop from a small group of cells. Konar and Nataraja (1965), Konar *et.al.* (1972), Mc. William *et.al.* (1974), Haccius (1978), Vasil and Vasil (1982), Wang and Vasil (1982) and Nomura and Komamine (1985) all suggest the origin of somatic embryos may be from single superficial cells, while Vasil and Hildebrandt (1966) and Pence *et.al.* (1979, 1980) points out that these embryos might arise from both single cell and multiple cell budding. In the process of indirect somatic embryogenesis from suspension or callus cultures, somatic

embryos which developed from a compact clump of embryogenic cells (proembryonal complex) arise from either single cells or by multiple-cell budding (Maheswaran and Williams, 1985).

In discussing somatic embryo development, it is important to consider the issue of totipotency, competence, determination and differentiation of cells. Haberlandt (1902) stated that a single plant cell has the capacity to behave as a zygote and develop to form the complete plant and that the recovery of the whole organism from single cultured cells via a process of embryogenesis would be the best proof of the totipotency of cells. However, as already discussed, the assumption of totipotency should be restricted to those functionally diploid cells which are not irreversibly differentiated and, even then, it might not be possible with present technology to make a practical demonstration of the state.

Competence and determination are developmental states, and as such are defined and assayed by the response of cells or tissues to external stimuli (Christianson, 1981). These two states undoubtedly exist in higher plants, although it is not completely clear that such states exist at the level of individual cells (Henshaw *et.al.*, 1982). Competent cells are defined as having the ability to respond to a particular inductive agent and, in doing so, they become determined for a particular developmental fate which will be attained, given appropriate permissive conditions (Waddington, 1977). If one accepts that these definition apply to plant cells, it can be assumed that the development of a set of manipulations to produce somatic embryos in a given species, hinges on the existence of embryogenically competent cells. Here it should be emphasized that the state of "embryogenic competence" which is defined operationally in terms of a particular sets of inductive conditions and a response, is not the same as the state of "totipotency", the definition of which does not exclude conditions yet to be discovered. From the

practical point of view, therefore, reference to the latter state is less usefull. In addition to its practical importance, it can be concluded that somatic embryogenesis is an ideal system for studying the theories of totipotency, competence, determination and differentiation of cells.

Abnormalities in cell division, cell differentiation and other developmental states can result in the formation of abnormal somatic embryos, which is one of the problems associated with the use of these systems. It has been suggested that imbalances in the culture medium used in the final subculture or unsuitable environmental conditions might be involved (Ammirato and Steward, 1971; Arrilaga *et.al.*, 1987). Many abnormal forms of somatic embryos can be found (Halperin, 1966; Ammirato and Steward, 1971; Konar *et.al.*, 1972; Ammirato, 1985) e.g. : (1) accessory or secondary embryos may occur along the whole embryogenic axis, apparently as a result of the cells within the small proembryos retaining their embryogenic competence and, rather than participating in the coordinated growth of the one embryo, becoming themselves embryogenic growth centres; (2) twin, triplet and multiple clusters of embryos, which generate multiple shoots when plantlets form, can be a consequence of the size of the cotyledonary ring being altered by too many cell divisions and/or premature cell enlargement, so that more than two centres can be initiated to form cotyledons; (3) poor development of cotyledons, single, multiple or fused cotyledons are probably caused by too few cell divisions or by the continuation of cell division in the cotyledonary ring even after the cotyledonary primordia are initiated; (4) embryos which fail to produce a normal shoot with leaves and stem can most likely be attributed to the malformation of the shoot apical meristem; (5) embryos which germinate precociously and produce malformed or aborted plants (Kerns, 1986; Ammirato, 1987; Arrilaga *et.al.*, 1987).

1.2.2. Application of somatic embryogenesis

The process of somatic embryogenesis has potential application in relation to propagation and breeding. For propagation purposes it is possible that the process might be used to produce large numbers of genetically uniform plants, assuming either that the cells in the embryogenic cultures are homogenous or that the embryos are derived from a uniform population of cells within the culture. Embryogenic suspension cultures could be particularly suitable for large-scale production and automation and the embryos are likely to have a number of advantages as propagules which might be encapsulated for field delivery. From the plant breeding point of view, embryogenic suspensions could provide the means by which plants are regenerated from cells which have been selected or otherwise manipulated in cultures. Without a suitable regeneration procedure, these *in vitro* approaches to breeding will be without value and in some species, such as cassava, somatic embryogenesis at present represents the only reasonably reliable approach to this problem. In particular, most genetic transformation techniques are heavily dependent on the availability of an efficient regeneration procedure.

High frequency of plant regeneration via somatic embryogenesis of some economically important crops has already been reported (Litz and Conover, 1983; Nabors *et.al.*, 1983; Ram and Nabors, 1984; Galiba and Yamada, 1988). Many species from different families of higher plants including both dicotyledons and monocotyledons have been showed to be able to undergo somatic embryogenesis, including *Pisum sativum L.* (Jacobsen and Kyseley, 1984), *Carica papaya L.* (Chen *et.al.*, 1987), *Pseudotsuga menzeiesii* (Mirc. J. Franco) (Durzan and Gupta, 1987), *Helianthus annuus L.* (Finer, 1987), *Theobroma cacao L.* (Kononowicz *et.al.*, 1988), *Persea americana* Mill.

(Alfaro and Murashige, 1988), *Cucumis sativus L.*, (Bergervoet *et.al.*, 1989), *Musa acuminata* (Escalant and Teisson, 1989), and *Albies alba* (Lang and Kohlenbach, 1989).

Plant regeneration from mechanically isolated, fully differentiated mesophyll cells of *Macleaya cordata* has been obtained by Kohlenbach (1965), and Lang and Kohlenbach (1975, 1978). Button and Bohta (1975) have also achieved regeneration through somatic embryogenesis by culturing single cells of citrus obtained from macerated callus.

Plant regeneration from isolated protoplast of higher plants via somatic embryogenesis has also been reported (Dudits *et.al.*, 1976; Kao and Michayluk, 1980; Santos *et.al.*, 1980; Arcioni *et.al.*, 1982; Lu *et.al.*, 1982; Xu *et.al.*, 1982; Davey and Cocking, 1983; Lu *et.al.*, 1983; Santos *et.al.*, 1983; Neidz *et.al.*, 1985). The development of somatic embryos derived from protoplasts isolated from embryogenic suspension cultures of *Pennisetum americanum* (Vasil and Vasil, 1980), *Pennisetum purpureum* (Vasil *et.al.*, 1983), *Picea abies* (Hakman *et.al.*, 1985), *Picea glauca* (Hakman and Fowke, 1987) and *Pinus taeda* (Gupta and Durzan, 1987) has also been reported.

1.3. CRYOPRESERVATION

In vitro systems are proving to be particularly useful for the long-term storage of germplasm of vegetatively propagated crop species, such as potato, cassava, yam and sweet potato in which there is a need to conserve clonal material (Henshaw,1982). They provide a particularly valuable alternative to conventional methods of germplasm

storage, which involve the maintenance of plants under field conditions where they are exposed to pests, diseases and unfavourable environmental conditions (Henshaw, 1982).

Among the various *in vitro* systems that are available, only shoot-tip cultures, as used for micropropagation, seem to have, whilst growing, the inherent genetic stability that is essential for these purposes. However, as long as they are growing, there is still some risks of long-term genetic drift with these cultures and alternative methods, involving non-growing *in vitro* systems are now being sought.

It is not easy to maintain cultures on a long-term basis under non-growing conditions and it is believed that cryopreservation techniques, involving storage at the temperature of liquid nitrogen (-196^{o}C), provide the only practical solution to the problems (Withers, 1985). Although there should be a high level of genetic stability in cryopreserved cells, it would still be important to use an inherently stable system, to reduce the risk of genetic change during the growth periods before and after storage. Shoot meristems would seem, therefore, to be suitable for the purpose and they have been used succesfully with a number of species, such as potato (Henshaw *et al.*,1985a,b). Some success has been achieved with shoot meristems of cassava (Kartha, 1985) but they do not seem to respond as readily as potato meristems to the cryopreservation procedures (Henshaw, pers.comm.) and it is important to investigate the suitability of alternative systems, such as somatic embryos, for this purpose. Also it would be extremely useful if stocks of cryopreserved somatic embryos or embryogenic tissues could be maintained on a long-term basis in order to provide a ready supply of experimental material, particularly for genetic transformation studies.

Cryopreservation techniques generally involve a number of stages : (1) excision of the organs to be frozen, (2) treatment of the organs with a cryoprotectant to aid preservation of the components during exposure to low temperatures, (3) freezing of the organs, which may be carried out at different cooling rates, down to the temperature of liquid nitrogen (-196^{o}C).

Since ice formation is one of the major causes of cellular damage during cryopreservation, the water content of cells -which varies according to the cell type and the state of differentiation- can be critical (Henshaw, 1982). In addition, therefore, to choosing an appropriate cryopreservation protocol (see Chapter 8), it is important to choose a suitable type of cell with a low water content. Next to the dehydrated cells in certain seeds and pollen grains, meristematic cells appear to be particularly suitable for cryopreservation (Henshaw and O'Hara, 1982) and, therefore, it might be anticipated that young somatic embryos and embryogenic tissues would respond favourably.

A further problem, however, with the cryopreservation of organized structures such as meristems and embryos is that, although individual cells might survive, there can be an overall loss of organization and possibly a loss of totipotency. Nevertheless, plants have been regenerated from cryopreserved meristems of *Solanum tuberosum* (Bajaj, 1985; Henshaw *et al.*, 1985b), of *Manihot esculenta* (Kartha, 1985), of *Dianthus caryophyllus* (Seibert, 1976); and from cryopreserved somatic embryos of *Citrus sp.* (Marin and Duran-villa, 1988) and of oil palm (Engelmann and Dereudrre, 1988).

1.4. GENETIC STABILITY OF CULTURES

The long-term maintainance of genetic integrity is essential if the goal of somatic embryogenesis is clonal reproduction. Ammirato (1983) and Flick *et.al.*, (1983) state that the method of maintenance of cultures can determine whether a culture retains its organogenic potential. A key factor in maintaining morphogenic potential is the maintenance of chromosome stability, and it has been suggested that this might be achieved if the cultures are subcultured frequently so that aneuploid cells do not accumulate as a result of alternating periods of nutrient starvation (Bayliss, 1977; Sunderland, 1977; Evans and Gamborg, 1982).

Even this strategy, however, is not a guarantee that the cultures will be genetically normal. Franklin *et.al.*, (1989), for example, showed that cells with higher ploidy were produced within three weeks of callus initiation and, therefore, it is apparent that as early as in the first days of culture an explant may comprise a heterogenous cell population. This heterogeneity might partly reflect a pre-existing condition (e.g. diploid and polyploid cells in a polysomatic species) and partly result from nuclear processes at the time of callus induction (chromosome endoreduplication, mitosis subsequent to nuclear fragmentation).

The explant tissue or starting material itself, even if it is not polysomatic, is believed to be one of the causes of abnormalities. Kudirka *et.al.*, 1986 point out that explant tissue coming from differentiated tissue may contain cells expressing different genes and these tissue-specific genes may continue to be expressed *in vitro*.

The chromosome instability could be induced by the *in vitro* conditions. The evidence is extensive that changes in chromosome number and in karyotype are

associated with *in vitro* culture (Vazquez and Ruiz, 1986). Zeng and Ouyang (1980) state that the *in vitro* culture method can also induce chromosomal abnormalities by breaking and altering at the fragile structural level. It, therefore, seems that certain genotypes are more unstable than others during *in vitro* cultures. This point of view is supported by Hu Han (1986) stating that cultured cells both animal and plant tissues are characterized by instability of chromosome number and structure, and by Kasha (1982) stating that the meristem-like cells that are in division tend to be more stable than more differentiated cells.

Plant growth regulators, in particular 2,4-D, can exercise upon the mitotic process (Mohandes and Grant, 1972; Bayliss, 1973), although some authors deny that 2,4D induces mitotic irregulaties (Singh and Harvey, 1975). Genetic variation which was detected by examining the progeny of regenerated plants from tissue cultures of barley occurred at 18.0 μM 2,4D for such traits as albinism, leaf shape and tiller fertility (Deambragio and Dale, 1980). Depending on the concentration and the exposure period, other plant regulators such as IAA, KIN and BA were also shown to cause chromosome number variation in *Vicia alba* cells (Ogura, 1982).

1.5. PROJECT AIM

The aims of project can be described under four headings: (1) to investigate an improved procedure for cassava somatic embryogenesis in order to obtain a higher frequency of response with a wider range of genotypes; (2) to establish a technique to maintain the production of cassava somatic embryos and embryogenenic tissues on a long-term basis; (3) to investigate the use of cryopreservation techniques for the long-term storage of cassava somatic embryos and embryogenic tissues; (4) to check

the genetic stability of the cassava embryogenic systems by counting the chromosomes of somatic embryos and of plants regenerated from somatic embryos.

An improved procedure for the induction and regeneration of cassava somatic embryogenesis in both semi-solid and liquid medium is necessary to obtain a higher frequency of embryogenesis with a wide range of cassava cultivars from both CIAT (Columbia) and IITA (Nigeria). In addition work with explants taken from different parts of the plant, suspension-culture and protoplast-culture procedures were also investigated. Factors affecting somatic embryogenesis, such as the composition of medium, subculture regimes, were varied.

As somatic embryos can be conveniently employed to initiate embryogenic tissues, it would be useful to have a regular supply of such embryos for use in a genetic transformation programme. It is likely that the only method that can be recommended for the long-term storage of the somatic embryos for this purpose would be based on a cryopreservation procedure involving storage at the temperature of liquid nitrogen ($-196^{o}C$). Thus, preliminary research to identify the critical stage at which somatic embryos are able to give the highest recovery rate, the optimal composition of medium and level of cryoprotectant was carried out. As well as a slow-cooling procedure, rapid cooling by direct immersion in liquid nitrogen was also investigated.

CHAPTER 2
MATERIALS AND METHODS

2.1. Plant materials

Clonal materials of cultivars CMC 40, CMC 76, MCol 22, MCol 113, MCol 216 and MCol 1684 received as stem cuttings from International Centre for Tropical Agriculture (CIAT), Cali, Columbia, were grown in the greenhouse and those of cultivars TMS-30040, TMS-30211, TMS-30555, TMS-30786, TMS-40160-P6, TMS-42025, TMS-50395, TMS-60142, TMS-60444, TMS-60506, TMS-63397, TMS-83350, TMS-84537, TMS-90059, TMS-90853, TMS-91934 received as meristem-tip cultures from IITA (Africa) were cultured *in vitro*.

2.2. Plant growth and maintenance in the greenhouse

Stem cuttings 15-20 cm in length were dipped into Seradix (May and Baker, Ltd.), a commercial hormone rooting powder, and planted in 16 cm diameter plastic pots containing Levington compost. The plants were subjected to a minimum temperature of 28^{o}C and a minimum twelve-hour photoperiod, using supplementary lighting when necessary.

The plants were cut back regularly, leaving a stem with 3-4 nodes above the compost surface for regeneration. This procedure was followed particularly after some parts of the plants had been taken for experimental work.

White fly and red spider mites were controlled by regular fumigation with Tedion (Midox) and Dicofol (Octavius Hunt). Several other pesticides i.e. Pynosect (Mitchell Cotts Chemicals), Malathion (ICI), Gamma Col (ICI) and X-All-Insecticides (Synchemicals) were also used to control white fly and green fly.

2.3. Shoot culture initiation and maintenance

Shoot cultures were subcultured every 4 months by placing 2-3 shoots possessing one axillary bud or apical meristems in 30 ml plastic vials (Sterilin, Ltd.) containing hormone-free medium supplemented with 2% sucrose.

2.4. Sterilisation of explants

Explants collected from plants in the greenhouse were surface sterilised by immersion in 5% sodium hypochlorite for 3 minutes and washed 3-4 times in sterile water.

Using a stereomicroscope (Olympus, Japan), sterile forceps and a hypodermic needle (Sabre International Products Ltd.), leaf lobes were removed from clonal tissues. All sterilisation procedures were carried out in 60 ml plastic vials (Sterilin Ltd.) and were performed in the sterile environment provided by a laminar flow cabinet (Microflow Pathfinder Ltd.)

2.5. Culture Media

Unless otherwise stated, the basal medium used was that of Murashige and Skoog (1962) supplied by Flow Laboratories (Scotland) and stored at $4^{o}C$. The media were supplemented with 2% sucrose (Kartha et.al.,1974) and plant growth regulators and were made up with double-distilled water. Heat-stable growth regulators and other

compounds were added before the medium was autoclaved, while heat-labile compounds (tryptophan, casein hydrolysate, proline, glutamine, and enzymes) were filter-sterilised through a membrane filter of pore size 0.2 (Sartorius Ltd.) and added when the media were still warm. All media were autoclaved for 15 minutes at 15 $lb.in^{-2}$ (1.87 bar) at 120°C. All Murashige and Skoog media were adjusted to pH 5.7 with 1/0.1 M sodium hydroxide or 1/0.1 M hydrochloric acid. Media were solidified with 0.6% w/v Oxoid No. 3 agar which was added after pH adjustment. Liquid sterile media were stored at 4°C, while the solid media were stored at room temperature.

2.6. Culture Vessels

Unless otherwise stated, clonal plant tissues were cultured on 20 ml semi-solid medium in 9 cm diameter plastic Petri dishes (Sterilin Ltd.). These Petri dishes containing the same amount of medium were also used for regenerating somatic embryos before separation, while 60 ml plastic vials (Sterilin, Ltd.) containing 30 ml semi-solid medium and 50 ml glass test tubes (Pyrex) containing 10 ml liquid medium were used for regeneration of individual somatic embryos and hardening plantlets before they were transferred to the greenhouse. 5 cm plastic Petri dishes containing 10 ml medium were used for culturing tissues before and after cryopreservation. 250 ml Erlenmeyer flasks placed on an orbital shakers were used for suspension cultures.

Petri dishes were sealed with Parafilm M. (American Can Co.), while plastic vials and glass test tubes were covered with plastic or aluminium caps.

2.7. Culture incubation conditions

Semi solid cultures were incubated at 25+1^{o}C and under continuous light with a 16 hour photoperiod (30 $Mm^{-1}s^{-1}$PAR) either in incubators (A. Gallenkamp and Co., Ltd.) with horizontal illumination provided by warm white fluorescent lamps (GEC, SK), or in a culture room.

Liquid cultures were incubated under the same conditions on an orbital shaker incubator (A. Gallenkamp and Co., Ltd.).

2.8. Staining for cell and protoplast viability

One drop of 5.0 mg fluorescein diacetate (FDA) solution in 1.0 ml acetone dissolved in 5 ml MS liquid medium, was added to one drop of cell or protoplast suspension on a glass slide. Samples were then observed under the fluorescence microscope with blue light.

2.9. Staining for protoplast cell walls

One drop of 0.6% (w/v) Calcoflour White in water was added to one drop of protoplast suspension on a glass slide. The stain was allowed to develop for a few minutes before the sample was observed under the fluorescence microscope with ultra violet light.

2.10. Light microscopy

The procedure used for fixation, paraffin embedding, and staining was a modification of Johansen (1940).

Specimens were fixed in FAA for at least eighteen hours at 4°C. They were then passed through a TBA series (Table 2.1)

Table 2.1. Summary of dehydration procedure

TBA	Water	95%ethanol	TBA	100%ethanol	Duration (at room temperature)
1	50	40	10	0	1 hour
2	30	50	20	0	overnight
3	15	50	35	0	1 hour
4	0	0	75	25	1 hour

Specimens were transferred to a saturated solution of Erythrosin B made up in pure TBA and left for 3-4 hours on top of oven at 70-80^{o}C. After a change to pure TBA, they were left overnight on oven top.

Wax chips (paraffin wax with ceresin) were added to the specimens in TBA. Vials containing 3-5 specimens were placed inside the oven at 70-80^{o}C and the TBA was allowed to evaporate. After 4-5 hours, the wax was removed and replaced with fresh molten wax three times, allowing 3-4 hours per wax change. The specimens and molten wax were poured into a prepared mould on a hot plate and the specimens were orientated with forceps. The surface of wax blocks was allowed to cool before immersion in cold water.

The specimens were sectioned at 6 um with microtome. Sections were floated on warm water to allow expansion and placed on glass slides smeared with Haupt's adhesive (1.0 g gelatin, 2.0 g phenol and 15 ml glycerol dissolved in 100 ml distilled water at 60^{o}C. A few drops of 3% formalin were around the sections to allow further expansion.

The slide was immersed in three Histoclear baths (three minutes each) and then Histoclear/ethanol (50:50) for three minutes before being taken through an ethanol series (100%, 95%, 70%, 50%, 30% for 3 minutes each) and then to distilled water for one minute.

The slide was immersed in a 1% (w/v) aqueous solution of toluidine blue for 45-60 seconds.

The slides were washed in distilled water for two minutes and then passed through an ethanol series (one minute each in 30%, 50%, 70%, 95% and 100%), Histoclear/ethanol (50:50) for one minute. Finally, three washes in Histoclear (3-5 minutes each). Specimens were then mounted on slides with DPX-mount.

2.11. Scanning Electron Microscopy

Specimens were fixed overnight in 4% glutaraldehyde in 0.2 M Sorensens phosphate buffer pH 7.0 at 4°C. Having been washed twice (for 10 minutes each) in phosphate buffer supplemented with 2% (w/v) sucrose, specimens were subjected to one change of 1% (v/v) osmium for one hour at room temperature. Specimens were then washed once again before they were soaked in 1% (v/v) tannic acid at room temperature. The osmium (OSO)-tannic acid tratment (Katsumoto *et.al.*, 1981) was used to avoid the shrinkage of plant cells. The specimens were dried using the CPD (Critical Point Dry) method after they had been dehydrated through a graded series of ethanol solutions. The dried samples were then coated with gold and they were examined with T-330 JEOL Scanning Electron Microscope.

2.12. Photography

Photomicrographs were taken with either an Olympus BH-2 (Japan) or Zeiss stereo microscope with attached Olympus OM-2 camera. Photographs of cultures were either taken with an Olympus OM-2 camera using either Kodachrome 64 or Kodak Panatomix X film.

CHAPTER 3

INDUCTION OF SOMATIC EMBRYOGENESIS IN CASSAVA

3.1. INTRODUCTION

3.1.1. General Pathways of Induction of Somatic Embryogenesis

There have been various attempts to categorize the embryogenic response of plant somatic tissues under *in vitro* conditions. Sharp *et.al.*(1982), for example, distinguished between two types of embryogenic response: "direct embryogenesis" in which the already determined cells (PEDC's, pre-embryogenic determined cells) responding to permissive conditions divide to produce somatic embryos without an intervening callus phase, and "indirect embryogenesis" in which differentiated cells are stimulated to divide and re-differentiate to form determined cells (IEDC's, induced embryogenic determined cells), from which the somatic embryos develop under suitable conditions. On the other hand, the model proposed by William and Maheswaran (1985), which might be more applicable to cassava, proposes a sequence of events during the development of a plant during which there is a progressive loss of embryogenic competence: initially all of the cells are embryogenically competent and they respond to inductive conditions in a coordinated manner, producing somatic embryos by a budding process; subsequently, as the plant matures, more localized regions of competence might proliferate before producing embryos by the budding process or isolated competent cells might develop directly into embryos. Stamp's (Stamp and Henshaw, 1982) observations indicated that the somatic embryos formed from the cotyledons of mature zygotic embryos of cassava are generally produced by a budding process from cells which have proliferated in an organized manner; the possibility that some of the embryos might have arisen directly from competent cotyledonary cells is not, however, completely excluded. As the explants also produce a rapidly growing true callus tissue which is very friable and apparently non-embryogenically competent, there remains the possibility that this tissue, under the right conditions, might be induced to re-differentiate to the competent state. In

that case, the cells would become IEDC's, in the sense of Sharp et al (1982), and the embryogenic pathway would be indirect. Normally, however, it seem likely that competent cells in the cotyledons proliferate clonally to produce a competent tissue which then produces the embryo by budding.

The induction treatment appears to foster dedifferentiation in the cells and also to induce embryogenic development. Ammirato (1987) points out that there is usually a one-to-one correspondence between the number of proembryos in the inoculum and the number of somatic embryos that mature when the small proembryos are moved from the auxin-containing to auxin-free or auxin-reduced medium. Work with alfalfa (Walker *et.al.*, 1979) indicated that embryogenic cells could only be induced if the cells were moved to medium containing containing 2,4-D and the embryos developed when the cells were then transferred to a kinetin-NAA medium.

3.1.2. Factors Affecting the Induction of Somatic Embryogenesis

The structure and behaviour of somatic embryogenesis are affected by many factors which can affect individually or incorporation with other factors such as : (1) the composition of the culture medium which can be categorised into sources and levels of nitrogen, osmotic concentration, composition and level of growth regulators, and composition of other compounds like activated charcoal, (2) the state of medium, (3) gaseous exchange, (4) subculture regime, (5) culture vessels and (6) environmental conditions such as light and photoperiod.

3.1.2.1. Composition of culture medium

3 1.2.1.1. Sources and level of nitrogen

It has been shown by a number of workers that the sources of nitrogen in the medium affect *in vitro* embryogenesis, in ways which are dependent upon the species used (Halperin, 1965; Reinert *et.al.*, 1967; Tazawa and Reinert *et.al.*, 1967; Tazawa and Reinert, 1969; Veliky and Rose, 1973; Raghavan, 1976; Ronchi, 1984; Skokut, 1985; Haga and Sodek, 1987).

Recent work has pointed to the beneficial aspects of certain amino acids such as proline in generating somatic embryos in maize (Amstrong and Green, 1985) and improving the quality (i.e. ability to convert into plants) of alfalfa somatic embryos (Stuart *et.al.*, 1985). The effect of other amino acids and mixtures of amino acids such as glutamine, tryptophan, casein hydrolysate and yeast extract on embryogenesis have also been demonstrated. Litz *et.al.*, (1982) suggested that glutamine addition to the medium helped to induce somatic embryogenesis; in contrast, the addition of either glutamine or proline to the callus induction medium did not increase the frequency of embryogenic callus formation from stem explants. In work with *Abies alba*, Schuller *et.al.* (1989) also showed that glutamine in combination with casein hydrolysate was not necessary for obtaining embryogenic callus. Trolinder and Goodin (1988) found that casein hydrolysate did not enhance development in plated cultures of cotton, but did appear to aid in embryo germination. Wang *et.al.* (1987) used casein hydrolysate to regenerate plants induced through somatic embryogenesis of *Oryza perennis*.

3.1.2.1.2. Formulation of media

The general formulation of media shown to have a distinctive effect on somatic embryogenesis. Ling *et.al.*, 1983 argued that Murashige and Skoog medium (1962) is the most suitable medium for subculture and for high frequency of differentiation of the embryogenic callus in rice while Heller's medium is more suitable for inducing somatic embryogenesis. It was also shown that the effect of White's medium on embryogenesis was similar to that of Murashige and Skoog if extra KNO_3 was added to the former.

3.1.2.1.3. Carbohydrate

Increasing the osmotic concentration, either by elevating the sucrose concentration or by adding compounds, such as myo-inositol or sorbitol, have been shown to modulate development of somatic embryos (Ammirato and Steward, 1971). Lu *et.al.*, 1982 found that a combination of 0.5 mgl-1 2,4-D with a high concentration of sucrose (12%, w/v) was the most suitable for induction of embryogenic callus in corn. Work with woody fruit trees also showed that a combination of 2,4-D and 6% (w/v) sucrose was the optimal formulation for inducing embryogenic callus (Litz, 1980). Chandler and Beard (1983); Finer (1987) support the result that high sucrose medium can enhance somatic embryogenesis of sunflower.

3.1.2.1.4. Growth regulators

2,4-D is believed to be essential for the induction of somatic embryogenesis in some plant species. The work of Halperin (1966, 1970) showed that the frequency of somatic

embryo formation was found to be higher in the presence of 2,4-D alone and that further development of somatic embryos was inhibited in medium containing high concentration of 2,4-D. Since these reports, many investigators, reported the importance of 2,4-D used alone for the induction of somatic embryogenesis. Lippmann and Lippmann (1984), for example, demonstrated that medium containing 2,4-D alone resulted in the highest frequency of somatic embryogenesis in soy bean. Tranfer to medium containing low concentration of 2,4-D, therefore, is essential for the maturation of somatic embryos (Raghavan, 1976; Ammirato, 1983). Cheema (1989) and van der Valk *et al.*(1989) showed that transfer to a low-2,4-D medium or to medium devoid of 2,4-D increased the number of somatic embryos formed and the number of somatic embryos regenerated to plantlets.

A combination of 2,4-D (or NAA) with cytokinin (zeatin-riboside or BA) was reported to be essential for the induction of some plant species (Kohlenbach, 1965; Lang and Kohlenbach, 1975, 1978; Kao and Michayluk, 1981; Ginger and Lineberger, 1989; Schuller *et.al.*, 1989).

Other growth regulators, such as GA_3 and ABA have also been found to affect embryo maturation (Ammirato, 1987). GA_3 has proved useful in a number of cases in encouraging embryo maturation or in stimulating the rooting and and subsequent growth of plants (Kochba *et.al.*, 1974; Lu *et.al.*, 1982). Rajaskaran and Mullins (1979) found that gibberellic acid treatment can substitute for chilling in the breaking of the somatic embryo dormancy which develops in certain species. ABA has proven useful in "normalising" somatic embryo development in caraway (Ammirato, 1974), carrot (Ammirato, 1983), pearl millet (Vasil and Vasil, 1981, 1982) and soy bean (Ranch *et.al.*, 1985). It was concluded that the sooner the ABA was applied to the caraway cultures, the more

normal was the somatic embryo maturation, and that the effects are quite specific with the time of application (Ammirato, 1983).

Other growth regulatory compounds such as dicamba and picloram have been shown to have an affect on somatic embryogenesis. Dicamba alone, added in SH medium, has proved capable of inducing embryogenic callus formation and somatic embryos in *Zea mays* (Conger *et.al.*, 1987), while dicamba in combination with a high concentration of sucrose was employed to maintain the embryogenic tissues. Jacobsen and Kysely (1984) using low concentrations of picloram, were able to obtain somatic embryos which were formed on the surface of leaf- derived callus of pea, upon transfer to liquid medium. This supports Beyl and Sharma (1983) that transfer of callus tissues to liquid media with low picloram concentration was successful in *Gasteria* and *Haworthia.*

It is quite clear, therefore, that growth regulators have a strong direct influence on somatic embryogenesis, but there are other medium additives which almost certainly affect the process indirectly through their effects on growth regulators. Activated charcoal, for example, is known to absorb strongly the different classes of growth regulators (Weatherhead *et al.*, 1979) and it is likely either to remove such compounds from the medium so that they are not available to the plant cells or, under circumstances where it has adsorbed large quantities it might slowly release them back into the medium. 1-4% activated charcoal in the auxin-free medium was used to restore the loss of embryogenic potential of carrot cultures (Fridborg and Eriksson, 1975). The fact that incorporation of 1% charcoal to the proliferation medium (with 2,4D) supported the development of embryos in the presence of comparatively high concentration of exogenous auxin strongly suggest that the loss of embryogenic potential in this system could have been due to the increased endogenous level of auxin.

3.1.2.2. The State of Medium and Culture Vessel

Somatic embryogenesis has been induced successfully on both semi-solid and liquid media in a wide range of vessels of different sizes and with different levels of gaseous exchange with the external atmosphere. The aeration of liquid media is clearly critical and that the partial pressure of oxygen in the medium has a strong influence on somatic embryogenesis in carrot. Steward *et al.* (1958) advocated the use with embryogenic suspension cultures of carrot of slowly rotating nipple flasks, claiming that the gentle agitation was important, but Halperin and Wetherell (1964) obtained successful results with more vigorously rotating Erlenmeyer flasks on a rotary shaker. However, Ammirato (1983) showed that somatic embryos grown in Erlenmeyer flasks on a rotary shaker, where there is considerable agitation and aeration, were more abnormal than those grown in tumble- tubes which provided a more gentle agitation and aeration.

The availability, uptake, evolution and dispersion of various gases can affect somatic embryogenesis (Ammirato, 1983). Rajasekhar *et.al.*, (1971) reported evidence of an unidentified volatile inhibitor of cell division that accumulated in suspension culture under condition of low aeration.

3.1.2.3. Cell Density

The development of somatic embryos seems to be very much affected by the density of embryogenic cells in the suspension (Halperin, 1967; Street, 1977). Sieving and centrifugation to synchronize a cell population can severely reduce the population density so that the effect of low density becomes critical (Huber *et.al.*, 1978). It has been

shown, however, that somatic embryos could be grown below the "minimum effective density" by the use of conditioned medium (Hari, 1980) or by the application of certain growth regulators such as ZEA and ABA at suitable concentrations (Ammirato, 1983).

3.1.2.4. Explant

Other factors which have an vital effect on somatic embryogenesis are the type of explants and the subculture regime. Only certain regions of the plant body, conditioned by the physiological state of the plant from which the explant is taken in any given species, seem able to respond readily (Lu *et.al.*, 1982; Lu and Vasil, 1982). Within certain species different genotypes have shown varying potentialities for somatic embryogenesis. In combination with explants and growth regulators used, the subculture regime also plays an important role in the maintenance of embryogenic competence.

3.2. MATERIALS AND METHODS

3.2.1. Source of explants

Young leaf lobes, axillary buds and meristems were taken from the following cassava cultivars supplied by CIAT (International Centre for Tropical Agriculture, Colombia): CMC 40, CMC 76, MCol 22, MCol 113, MCol 216, MCol 1684 either propagated in the green house or in sterile vials (Sterilin, Ltd.) *in vitro* (see Section 2.3 for the details of growing conditions). They were also taken from the following cassava cultivars propagated *in vitro* on MS hormone-free medium supplemented with 2% (w/v) sucrose and supplied by IITA (International Institute of Tropical Agriculture, Nigeria): TMS-30040, TMS-30211, TMS-30555,TMS-30786, TMS-40160-P6, TMS-42025, TMS-50395, TMS-60142, TMS-60444, TMS-60506, TMS-63397, TMS-83350, TMS-84537,TMS-90059, TMS-90853, TMS-91934.

3.2.2. Culture procedure

Unless otherwise stated, explants excised from clonal tissues were cultured on semi-solid MS medium supplemented with 4.0 mgl^{-1} 2,4-D and were incubated for 14 days (Stage-I) before transfer to Stage II semi-solid hormone-free MS medium, until the somatic embryos had developed. Explant sterilisation and culture conditions were as described in Chapter 2.

3.2.3. Culture media

The basal medium used and the procedures of making media were as described in Chapter 2. Heat-labile compounds such as glutamine, proline, casein hydrolysate and

tryptophan were filter sterilised through a Sartorius membrane filter of pore size 0.2 (Sartorius Ltd) and added when the media were still warm.

3.2.4. Culture vessels

Unless otherwise stated, explants were cultured either on semi-solid medium in 9.0 cm plastic Petri dishes or in liquid medium in 5.0 cm Petri dishes (Sartorius Ltd.).

3.3. RESULTS

3.3.1. Induction of somatic embryogenesis on semi-solid medium

Factors affecting the induction of somatic embryogenesis on semi solid medium were investigated by altering the ratio of nitrate to ammonium ion in the medium used, employing different types of growth regulators or other compounds at different concentrations, and incubating the explants for different duration.

Unless otherwise stated, the cassava cultivar used was CMC 76. A range of sizes of leaf lobes was used and all explants were subjected to a two-stage culture procedure. The frequency of explants producing somatic embryos, the number of somatic embryos produced per explant and the total number of somatic embryos produced were scored.

3.3.1.1. The effect of 2,4-D on somatic embryogenesis with South American (CIAT) and African (IITA) cultivars

There were 5 cultivars from CIAT (Colombia) : CMC 40, CMC 76, MCol 22, MCol 113 and MCol 216, and 16 cultivars from IITA (Nigeria) : TMS-30040, TMS-30211, TMS-30555, TMS-30786, TMS-40160-P6, TMS-42025, TMS-50395, TMS-60142, TMS-60444, TMS-60506, TMS-63397, TMS-83350, TMS-84537, TMS-90059, TMS-90853, and TMS-91934 were used to investigate the effect of 2,4-D on somatic embryogenesis. Due to restrictions the availability of explants, three experiments related to 2,4-D were carried out separately : (1) leaf lobes of CMC 40, CMC 76 and MCol 113 were used to investigate the optimum concentration of 2,4-D and optimum duration of incubation in Stage-I medium, (2) leaf lobes, axillary buds and meristems of

apical buds of CMC 40, CMC 76, MCol 22, MCol 113 and MCol 216 were used to investigate the effect of 2,4-D, NAA and a combination of NAA and BAP on somatic embryogenesis, (3) leaf lobes and axillary buds of the African cultivars were cultured on 2,4-D containing medium to investigate their capability of undergoing somatic embryogenesis.

3.3.1.1.1. Effect of the size of leaf lobes, concentration of 2,4-D and period of incubation in Stage-I medium on somatic embryogenesis of cultivars CMC 40, CMC 76 and MCol 113

Two ranges of size of leaf lobes (1-3 mm and 3-5 mm in length) were cultured on MS medium containing four different concentrations of 2,4-D (2.0, 4.0, 6.0, and 8.0 mgl^{-1}) and were incubated for either 5, 8, 13, and 17 days before they were transferred onto the hormone-free Stage-II medium for 21 days. The frequency of explants producing somatic embryo and the number of somatic embryos produced per explant were scored and displayed in Table 3.1.

Leaf lobes of CMC 40 and of MCol 113 failed to undergo embryogenesis irrespective of the concentrations of 2,4-D and the duration of incubation on Stage-I medium. Depending on the CMC 76 growing on medium supplemented with 2.0, 4.0, or 6.0 mgl^{-1} 2,4-D were able to undergo embryogenesis. This frequency was achieved by incubating explants either for 17 days on medium containing 2.0 mgl^{-1} 2,4-D, for 13 days on medium with 4.0 mgl^{-1}, or for 9 days on medium with 6.0 mgl^{-1} 2,4-D. It seemed, therefore, that a longer period of incubation was required if the lower concentrations of 2,4-D were to be used used to obtain the highest frequency of embryogenesis. Medium with 4.0 mgl^{-1} 2,4-D, however, seemed to be the best in terms of the production of total somatic embryos. This medium also resulted in the highest frequency of leaf lobes 1-3

Table 3.1. The effect of the size of leaf lobes, concentration of 2,4-D and period of incubation in Stage-I medium on somatic embryogenesis with cassava cultivar CMC 76

Level of 2,4-D (mgl^{-1})	period of incubation in Stage-I medium (days)	size of leaf lobes (mm)	%explants forming foliose structures	%explants producing somatic embryos	average number of somatic embryos/ explant	total somatic embryo product-ion
2.0	5	1-3	10	0	0 ace	0
		3-5	30	0	0 ace	0
	9	1-3	20	10	1.0 bce	2
		3-5	50	10	1.0 bde	2
	13	1-3	20	10	2.0 bcf	4
		3-5	30	40	3.0 bdf	24
	17	1-3	40	20	5.0 bcf	20
		3-5	60	50	5.4 bdf	54
4.0	5	1-3	30	0	0 ace	0
		3-5	50	0	0 ace	0
	9	1-3	20	20	2.0 bce	8
		3-5	60	40	5.0 bde	40
	13	1-3	20	50	6.0 bcf	60
		3-5	30	60	6.0 bdf	72
	17	1-3	10	30	3.7 bcf	22
		3-5	20	40	6.3 bdf	50
6.0	5	1-3	40	0	0 ace	0
		3-5	60	0	0 ace	0
	9	1-3	10	30	2.7 bce	16
		3-5	60	60	5.7 bde	68
	13	1-3	10	20	1.5 bcf	6
		3-5	30	30	3.0 bdf	18
	17	1-3	10	20	4.0 bcf	16
		3-5	10	40	6.0 bdf	48

Table 3.1. (continued)

Level of 2,4-D (mgl^{-1})	period of incubation in Stage-I medium (days)	size of leaf lobes (mm)	%explants forming foliose structures	%explants producing somatic embryos	average number of somatic embryos/ explant	total somatic embryo product-ion
8.0	5	1-3	40	0	0 ^{ace}	0
		3-5	50	0	0 ^{ace}	0
	9	1-3	20	30	2.3^{bce}	14
		3-5	60	30	3.3^{bde}	20
	13	1-3	10	10	4.0^{bcf}	8
		3-5	10	10	8.0^{bdf}	16
	17	1-3	0	10	2.5^{bcf}	5
		3-5	20	20	7.5^{bdf}	30

Values with same superscript do not differ significantly ($P<0.05$)

Key :

The above results were obtained with cultivar CMC 76; no somatic embryos were produced by cultivars CMC 40 and MCol 113.

Number of replicates : 20

Basal medium : MS supplemented with 2% (w/v) sucrose

Regeneration medium : hormone-free MS medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\,\mu Mm^{-2}s^{-1}$ PAR

Plate 3.1.

Induction and regeneration of somatic embryos from leaf-lobes explants cultured on semi solid MS medium

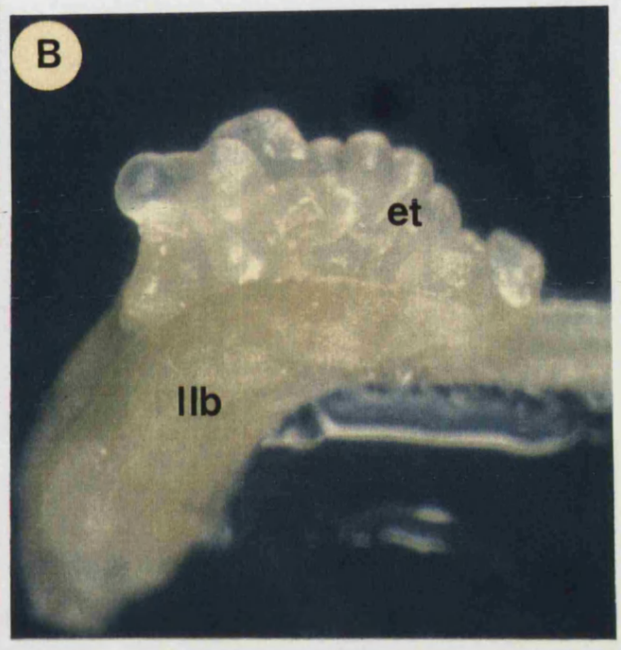
A. Size of immature leaf lobes used for the induction of somatic embryogenesis. Scale unit = 1 mm

B. Induction of somatic embryogenesis from 1-3 mm immature leaf lobes of cassava cultivar CMC 76 cultured on semi-solid medium containing 4.0 mgl^{-1} 2,4-D for 9 days. x40.5.

C. Regeneration of a clump of somatic embryos of CMC 76 on hormone-free medium. x25.

D. Induction of somatic embryos of CMC 40, CMC 76. MCol 22 and MCol 113 on medium supplemented with 9.0 or 12.0 mgl^{-1} picloram. x4.5.


A


B
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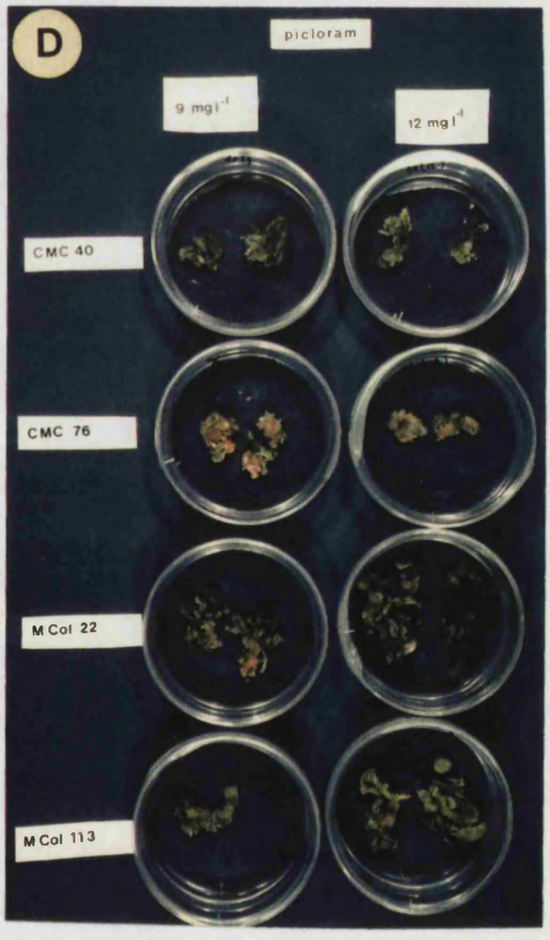

D
picloram
9 mgl-1
12 mgl-1
CMC 40
CMC 76
M Col 22
M Col 113

mm in length producing somatic embryos (50%) when they were incubated for 13 days. Plate 3.1B,C show the formation of somatic embryos from leaf lobes of cassava cultivar CMC 76 cultured on MS semi-solid medium supplemented with 4.0 mgl^{-1} 2,4-D.

Five days incubation, irrespective of the concentrations of 2,4-D used, seemed to be insufficient for the induction of somatic embryos. This period of incubation led to a high frequency of explants producing foliose structures. Plate 3.1A shows the range of sizes of leaf lobes and the induction of embryogenesis from leaf lobes. Plate 3.1 shows the range of sizes of leaf lobes and the induction of embryogenesis from leaf lobes.

3.3.1.1.2. Effect of 2,4-D, NAA and a combination of NAA and BAP on somatic embryogenesis with leaf lobes, axillary buds and meristems from South American cassava cultivars

Other types of explants such as axillary buds and meristems, were tested since in the previous experiment with several cultivars only the leaf lobes of CMC 76 were able to undergo somatic embryogenesis. These explants of cultivars CMC 40, CMC 76, MCol 22, MCol 113 and MCol 216 were incubated for 14 days on MS medium supplemented with 4.0 or 6.0 mgl^{-1} 2,4-D, 4.0 mgl^{-1} NAA, or a combination of 0.1 mgl^{-1} NAA and 0.1 mgl^{-1} BAP before transfer to hormone-free MS medium. A comparison was also made between axillary buds taken from plants grown in the greenhouse and those taken from plants grown *in vitro* (see Section 2.3.). The results are displayed in Tables 3.2 and 3.3.

Only medium supplemented with 2,4-D resulted in somatic embryogenesis, while those with either NAA alone or a combination of NAA and BAP resulted in the formation of plantlets by organogenesis. All types of explants of CMC 76 produced somatic

Table 3.2. The effect of the size of leaf lobes and concentration of 2,4-D on the induction of somatic embryos from South American cassava cultivars

Cultivars	Length of leaf lobes (mm)	Level of 2.4D (mgl^{-1})	%explants producing somatic embryos	number of somatic embryos/ explant	total production of somatic embryos
CMC 40	1 - 3	4.0	0	0	0
	3 - 5		0	0	0
	5 - 9		0	0	0
	1 - 3	6.0	0	0	0
	3 - 5		0	0	0
	5 - 9		0	0	0
CMC 76	1 - 3	4.0	40	12	96
	3 - 5		30	11	66
	5 - 9		30	20	120
	1 - 3	6.0	40	3	24
	3 - 5		40	11	88
	5 - 9		0	0	0
MCol 22	1 - 3	4.0	50	4	40
	3 - 5		40	2	16
	5 - 9		10	3	6
	1 - 3	6.0	20	3	12
	3 - 5		0	0	0
	5 - 9		0	0	0
MCol 113	1 - 3	4.0	0	0	0
	3 - 5		0	0	0
	5 - 9		0	0	0
	1 - 3	6.0	0	0	0
	3 - 5		0	0	0
	5 - 9		0	0	0
MCol 216	1 - 3	4.0	0	0	0
	3 - 5		0	0	0
	5 - 9		0	0	0
	1 - 3	6.0	0	0	0
	3 - 5		0	0	0
	5 - 9		0	0	0

Key : Number of replicates : 20

Basal medium : MS supplemented with 2% (w/v) sucrose

Regeneration medium : hormone-free MS medium

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod,

$30 \mu Mm^{-2}s^{-1}$ PAR

Table 3.3. The effect of the type of explant on somatic embryogenesis with South American cassava cultivars

Type of explant	cultivar	% explants producing somatic embryos	average number of somatic embryos/ explant	total somatic embryo production
axillary buds	CMC 40	0	0	0
	CMC 76	50	10	50
	M Col 113	0	0	0
	M Col 216	0	0	0
meristems	CMC 40	0	0	0
	CMC 76	30	5	15
	M Col 113	0	0	0
	M Col 216	70	2	14

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

embryos, as well as leaf lobes of MCol 22 and meristems of MCol 216 (Tables 3.2 and 3.3). CMC 76, therefore, proved to be superior to the other cultivars in terms of the production of somatic embryos.

As with plantlets regenerated through somatic embryogenesis, those regenerated through organogenesis showed different growth rates betweeen cultivars. Plantlets of CMC 40 and CMC 76 grew faster than those of MCol 113 and MCol 216, especially when they were grown on medium with a combination of NAA and BAP.

It was also noted (Table 3.3) that axillary buds taken from plants grown *in vitro* underwent embryogenesis at a higher frequency than those taken from the greenhouse (30%-50% and 10%-20% respectively).

3.3.1.1.3. The effect of 2,4-D and type of explant on somatic embryogenesis with a range of African cultivars

The explants of the African cultivars were all taken from plants grown *in vitro* (see Section 2.3), since there were no plants grown in the greenhouse. Since it was not known which parts of plants could undergo embryogenesis, different sizes of leaf lobes, axillary buds, petioles, stems and roots of sixteen African cultivars were tested. All explants were cultured on medium supplemented with either 2.0, 4.0 or 6.0 mgl^{-1} 2,4-D or 4.0 mgl^{-1} NAA. Table 3.4 displays the results of this experiment.

Table 3.4. The effect of 2,4-D and type of explant on somatic embryogenesis with a range of African cultivars

Cultivar	% Explants producing embryogenic tissue		Number developed somatic embryos per explant	
	leaf lobes	axillary buds	leaf lobes	axillary buds
TMS-30040	40.0	10.0	2	1
TMS-30786	37.0	50.0	0	3
TMS-40160 -P6	30.0	30.0	2	2
TMS-42025	0	0	0	0
TMS-50395	25.0	10.0	0	1
TMS-60142	66.7	25.0	15	4
TMS-60444	40.0	40.0	0	4
TMS-83350	83.3	54.5	7	3
TMS-84537	100.0	20.0	23	2
TMS-90059	66.7	33.3	7	2
TMS-90853	28.6	10.0	1	0
TMS-60506	50.0	-	1	-
TMS-63397	40.0	-	1	-
TMS-30211	-	10.0	-	2
TMS-30555	-	12.5	-	1
TMS-91934	-	33.3	-	4

Key :

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

Fifteen out of the sixteen cultivars growing on medium supplemented with 4.0 mgl^{-1} 2,4D were able to produce somatic embryos, either from leaf lobes of TMS 60506 and TMS 63397, from axillary buds of TMS 30211, TMS 30555 and TMS 91934, or from both leaf lobes and axillary buds of TMS 30040, TMS 30786, TMS 40160-P6, TMS 50395, TMS 60142, TMS 60444, TMS 83350, TMS 84537, TMS 90059 and TMS 90853. Petioles, stems and roots of all cultivars tested were not able to undergo somatic embryogenesis. Leaf lobes in 3-5 mm size range gave a higher frequency of embryogenesis (up to 100% of those of TMS 84537) on medium supplemented with 4.0 mgl^{-1} 2,4-D. This cultivar also proved to be superior to the other cultivars in terms of the average number of somatic embryo produced per explant (23 embryos).

Morphogenetic differences were noted among the somatic embryos regenerated from certain cultivars; somatic embryos of TMS 84537 and TMS 90059 possessed multiple, curled cotyledons which eventually converted to normal plantlets following transfer to Stage-II medium, while those of TMS 60142 possessed long hypocotyls with fused cotyledons. It was also noted that the growth rates of somatic embryos from some of the cultivars was different, with the result that somatic embryos of TMS 83350 and TMS 90059, for example, were as large as 7-9 mm in size by day 30.

3.3.1.2. The effect of picloram and dicamba on somatic embryogenesis with a range of South American cultivars

Attempts were made to induce somatic embryogenesis from cultivars CMC 40 and MCol 113 which were considered to be recalcitrant cultivars since they had failed to undergo embryogenesis in experiments using the growth regulator 2,4-D. In these experiments, the growth regulators picloram and dicamba were used as an alternative to

2,4-D. As a comparison, other cultivars, such as CMC 76 and MCol 22, which had been shown to be responsive to 2,4-D (see Section 3.3.1.1.1) were also investigated. To investigate the optimum duration of incubation on the Stage-I medium, only explants of cultivar CMC 40 were used due to the shortage of explants from the other cultivars.

3.3.1.2.1. The effect of picloram on the induction of somatic embryogenesis with cassava cultivars CMC 40. CMC 76, and MCol 113.

Seven different levels of picloram (0.06, 0.60, 1.00, 3.00, 6.00, 9.00 and 12.00 mgl^{-1}) added to Stage-I medium were tested with two different size ranges of leaf lobes of cultivars CMC 40, CMC 76 and MCol 113. The frequency of leaf lobes producing somatic embryos and the average number of somatic embryos produced per explant were scored after they were cultured for 14 days on Stage-I and 21 days on Stage-II medium. Table 3.5 shows that the concentration of picloram to or higher than 1.00 mgl^{-1}, were effective in inducing embryogenesis from leaf lobes of cassava cultivar CMC 76.

Both CMC 40 and MCol 113 were able to undergo embryogenesis in response to picloram, but the concentrations of picloram required were higher than those required by CMC 76; both CMC 40 and MCol 113 required 6.00 mgl^{-1} to induce embryogenesis from 1-3 mm leaf lobes and higher than 6.00 mgl^{-1} to induce embryogenesis from 3-5 mm leaf lobes, while those of CMC 76 only required 1.00 mgl^{-1} picloram. The optimum picloram concentration for both CMC 40 and MCol 113 was 12.00 mgl^{-1}, while that for CMC 76 was 3.00 mgl^{-1} and 9.00 mgl^{-1} depending on the size of leaf lobes used. In the case of CMC 76, picloram at a concentration of 9.00 mgl^{-1} brought about the highest frequency of explants of 1-3 mm in length producing somatic embryos

Table 3.5. The effect of picloram on the induction of somatic embryogenesis with cassava cultivars CMC 76, CMC 40 and MCol 113.

cultivar	level of picloram (mgl^{-1})	size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total somatic embryo production
CMC 76	1.0	1-3	10	10 [ad]	10
		3-5	10	30 [ad]	30
	3.0	1-3	60	7 [ad]	42
		3-5	70	23 [ad]	161
	6.0	1-3	50	7 [ad]	35
		3-5	60	15 [ad]	90
	9.0	1-3	90	7 [ad]	63
		3-5	40	23 [ad]	92
	12.0	1-3	60	4 [ad]	24
		3-5	50	15 [ad]	75
CMC 40	1.0	1-3	0	0 [be]	0
		3-5	0	0 [be]	0
	3.0	1-3	0	0 [be]	0
		3-5	0	0 [be]	0
	6.0	1-3	20	2 [be]	4
		3-5	0	0 [be]	0
	9.0	1-3	20	2 [be]	4
		3-5	10	2 [be]	2
	12.0	1-3	30	3 [be]	9
		3-5	10	3 [be]	3

Table 3.5. (continued)

cultivar	level of picloram (mgl^{-1})	size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total somatic embryo production
M Col 113	1.0	1-3	0	0 be	0
		3-5	0	0 be	0
	3.0	1-3	0	0 be	0
		3-5	0	0 be	0
	6.0	1-3	10	5 be	5
		3-5	0	0 be	0
	9.0	1-3	20	1 be	2
		3-5	40	5 be	20
	12.0	1-3	40	22 be	88
		3-5	30	4 be	12

Values with same superscript do not differ significantly ($P<0.05$).

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Duration in induction medium : 20 days

Regeneration medium : hormone-free MS mediurn

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\ \mu Mm^{-2}s^{-1}$ PAR

Table 3.6. The effect of picloram and period of incubation on somatic embryogenesis with cassava cultivar CMC 76

Level of picloram (mgl^{-1})	size of leaf lobes (mm)	period of incubation on Stage-I (days)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
9.0	1-3	20	60	11	66
		30	60	15	90
	3-5	20	80	24	192
		30	70	18	126
12.0	1-3	20	70	11	77
		30	90	32	288
	3-5	20	80	33	264
		30	90	105	945

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Light conditions : 16 hours photoperiod,

$30 \mu Mm^{-2}s^{-1}$ PAR

Temperature : $25 \pm 1^{o}C$

Table 3.7. The effect of picloram and period of incubation on somatic embryogenesis with cassava cultivar CMC 40

Level of picloram (mgl^{-1})	size of leaf lobes (mm)	period of incubation on Stage-I (days)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
9.0	1-3	20	20	2^a	4
		30	50	12^b	60
		40	30	5^c	15
	3-5	20	10	2^a	2
		30	20	35^b	70
		40	30	3^c	9
12.0	1-3	20	30	3^a	9
		30	50	6^b	30
		40	30	10^c	30
	3-5	20	10	3^a	3
		30	10	36^b	36
		40	40	3^c	12

Values with same superscript do not differ significantly ($P<0.05$)

Key

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Temperature : 25 ± 1^0C

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

(90%) while a concentration of 3.00 mgl^{-1} caused 70% of explants of 3-5 mm in length to produce somatic embryos.

Somatic embryogenesis with cultivar CMC 40 would also seem to be affected by the duration of incubation on the Stage-I medium (Table 3.7); of the periods of incubation tested, 30 days was shown to be optimal, irrespective of the level of picloram tested (9.00 and 12.00 mgl^{-1}). This period resulted in increases in the frequency of explants producing somatic embryo up to values of 50% and in the average number of somatic embryos produced per explant up to 36. Plate 3.1D shows the regeneration of somatic embryos derived from leaf lobes of South American cassava cultivars growing on picloram containing medium.

3.3.1.2.2. Effect of dicamba on the induction of somatic embryogenesis with cassava cultivars CMC 40, CMC 76 and MCol 113

Unlike picloram (Section 3.3.1.2.1), the range of concentrations of dicamba tested were quite wide (1.0, 3.3, 10.0, 33.0 and 66.0 mgl^{-1}) due to the fact that explants of the cultivars tested did not seem to respond to dicamba at low concentrations. Two sizes of leaf lobes were incubated for 14 days on these media before they were tranferred to hormone-free medium for 21 days. Table 3.8 shows the results of this experiment.

Both cultivars CMC 40 and MCol 113 were not able to undergo embryogenesis when they were cultured on medium supplemented with dicamba at concentrations up to 10.0 mgl^{-1}. Of the cultivars tested, CMC 40 required the highest concentration of dicamba (66.0 mgl^{-1}) to be able to undergo embryogenesis, followed by MCol 113 which required 33.0 mgl^{-1} and then CMC 76 which only required 3.30 mgl^{-1}.

Table 3.8. The effect of dicamba on the induction of somatic embryogenesis with cassava cultivars CMC 76, CMC 40 and MCol 113.

Cultivar	Level of dicamba (mgl^{-1})	size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
CMC 76	1.0	1-3	0	0 adf	0
		3-5	0	0 aef	0
	3.3	1-3	20	12 adf	24
		3-5	0	0 aef	0
	10.0	1-3	20	6 adf	12
		3-5	10	3 aef	3
	33.0	1-3	60	10 adg	6
		3-5	40	5 aeg	20
	66.0	1-3	30	5 adg	15
		3-5	10	6 aeg	6
CMC 40	1.0	1-3	0	0	0
		3-5	0	0	0
	3.3	1-3	0	0	0
		3-5	0	0	0
	10.0	1-3	0	0	0
		3-5	0	0	0
	33.0	1-3	0	0	0
		3-5	0	0	0
	66.0	1-3	0	0	0
		3-5	10	6	6

Table 3.8. (continued)

Cultivar	Level of dicamba (mgl^{-1})	size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
M Col 113	1.0	1-3	0	0^{bdf}	0
		3-5	0	0^{bef}	0
	3.3	1-3	0	0^{bdf}	0
		3-5	0	0^{bef}	0
	10.0	1-3	0	0^{bdf}	0
		3-5	10	3^{bef}	3
	33.0	1-3	30	5^{bdg}	15
		3-5	20	9^{beg}	18
	66.0	1-3	10	1^{bdg}	1
		3-5	40	4^{beg}	16

Values with same superscript do not differ significantly ($P<0.05$)

Key :

Number of replicates : 10

Basal medium : MS

Culture procedure : 20 days on induction medium and 21 days on regeneration medium (hormone-free MS medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

CMC 76 again proved to be superior to both cultivars CMC 40 and MCol 113 in terms of the frequency of explant producing somatic embryos and the average number of somatic embryos produced per explant. The frequency of explants of CMC 76 producing somatic embryo increased (up to 60%) in parallel with the increase of dicamba concentrations up to 33.0 mgl^{-1}, above which concentration dicamba caused decreases in the frequency of embryogenesis and the average number of somatic embryos produced per explant. A concentration of 33 mgl^{-1} dicamba, therefore, was optimal for the induction of embryogenesis of not only MCol 113 but also CMC 76.

3.3.1.3. The effect of inorganic and organic nitrogen compounds on somatic embryogenesis of cassava cultivar CMC 76

Glutamine, proline, tryptophan, as amino acid sources, and casein hydrolysate, as a mixture of amino acids were tested for their effects on cassava somatic embryogenesis. In addition, the effects of different ratios of ammonium and nitrate ions were also investigated since at present there are still a contradictory conclusions about whether nitrate or ammonium plays a more vital role in somatic embryogenesis. The basal medium used was that of Murashige and Skoog (1962), supplemented with 2% sucrose with alterations to the ratio of nitrate to ammonium.

3.3.1.3.1. The effect of the ratio of nitrate to ammonium ions on somatic embryogenesis with cassava cultivar CMC 76

In addition to the standard Murashige and Skoog medium which contains nitrate and ammonium ions at a molar ratio of 40 : 20, other ratios of nitrate to ammonium (20 : 20 and 20 : 40), and B5 standard medium which contains nitrate and ammonium at a ratio of 25 : 2 (as well as other differences in ion concentrations) were also tested. Leaf lobes were cultured on these modified medium containing 4.0 mgl^{-1} 2,4-D for 14 days before they were tranferred to the standard MS hormone-free medium. To investigate the relationship between these treatments and the size of leaf lobes, each treatment involved three different sizes of leaf lobes (1-3 mm, 3-5 mm and 5-9 mm in length). The frequency of explants producing somatic embryos, the average number of somatic embryos produced per explant, the frequency of large-sized, medium-sized and small-sized of somatic embryos produced, and the frequency of malformed somatic embryos are displayed in Tables 3.9 and 3.10).

The ratio of nitrate to ammonium ions was proved to be important in terms of the production of somatic embryo. Medium containing a ratio of nitrate to ammonium of 40 : 20 (i.e. the standard MS medium) which gave rise to the highest number of somatic embryos produced per explant (16.8 embryos), of total somatic embryos produced and of somatic embryos converted to normal plantlets was different from other media. On this medium, the responses of different sizes of leaf lobes also proved to be different; 1-3 mm leaf lobes seemed to be the best in terms of the production of somatic embryos, but 5-9 mm leaf lobes seemed to be the best if the somatic embryos were required to be converted to normal plantlets at a high frequency (Table 3.9).

Table 3.9. The effect of the ratio of nitrate to ammonium ions on the production somatic embryos derived from leaf lobes of cultivar CMC 76

Basal medium	Molar ratio of NO_3^-:NH_4^+	Size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos	%somatic embryos converting to normal plants
MS	40:20	1-3	48	16.8	202	8.9
		3-5	56	9.6	134	11.2
		5-9	28	12.9	90	15.5
	20:20	1-3	24	4.3	26	3.8
		3-5	52	4.5	59	0
		5-9	20	3.2	16	12.5
	20:40	1-3	56	9.0	126	9.5
		3-5	20	16.8	84	9.5
		5-9	8	3.0	6	0
B5	25:2	1-3	52	7.0	91	5.5
		3-5	12	3.3	10	10.0
		5-9	12	8.3	25	16.0

Key :

Number of replicates : 20

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$PAR

Table 3.10. The effect of the ratio of nitrate to ammonium ions on the size of somatic embryos derived from leaf lobes of cassava cultivar CMC 76

Basal medium	Molar ratio of NO_3^-:NH_4^+	Size of leaf lobes (mm)	% large sized somatic embryos[1]	% medium sized somatic embryos[2]	% small sized somatic embryos[3]	% malformed somatic embryos
MS	40:20	1-3	25.1	58.6	16.3	86.2
		3-5	36.7	10.5	31.2	80.2
		5-9	38.9	26.7	34.4	85.6
	20:20	1-3	23.1	53.8	23.1	88.5
		3-5	54.2	27.1	18.6	76.3
		5-9	68.7	31.2	0	93.7
	20:40	1-3	25.4	37.3	37.3	91.3
		3-5	27.4	45.2	45.2	86.9
		5-9	33.3	33.3	33.3	83.3
B5	25:2	1-3	31.2	39.1	29.4	93.5
		3-5	40.0	0.0	60.0	100.0
		5-9	16.0	16.0	68.0	100.0

Key :

Number of replicates : 20

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

The ratio of nitrate to ammonium not only affected the production of somatic embryos, but also the normality of somatic embryos produced. Explants growing on medium containing an equal ratio of nitrate to ammonium ions (20 : 20) gave rise to the highest frequency of somatic embryos possessing pale and malformed cotyledons (93.8%) especially among those regenerated from 5-9 mm leaf lobes (Table 3.10).

Another effect of the ratio of nitrate to ammonium ions was on the growth of the somatic embryos produced; the highest frequency of large-sized (8-12 mm in size) somatic embryos (68.8%) was obtained by culturing 5-9 mm leaf lobes on medium containing a 20 : 20 ratio (Table 3.10).

In general, Murashige and Skoog medium was superior to B5 medium in terms of the production of somatic embryos and of normal plantlets (Table 3.9). The total number of somatic embryos produced by explants growing on Murashige and Skoog medium was in the range 90-202, while that produced by explants growing on B5 medium was only in the range 10-91.

3.3.1.3.2. The effect of organic nitrogen on cassava somatic embryogenesis

A range of concentration of tryptophan and casein hydrolysate added either to both Stage I and Stage II, or only Stage II was tested to investigate their effects on the induction of somatic embryogenesis. Proline and glutamine alone or in combination were also tested. Unless otherwise stated, the pH of amino acid stock solutions was adjusted to 5.7 before they were filter sterilised and added into warm autoclaved either Stage-I or Stage-II medium. Three different ranges of size of leaf lobes (1-3, 3-5, 5-9 mm) were employed in tryptophan and casein hydrolysate treatments, while only one

size of leaf lobes used to investigate the effects of proline and glutamine because of the availability of explants.

3.3.1.3.2.1. The effect of tryptophan and casein hydrolysate in somatic embryogenesis of cassava cultivar CMC 76

Tryptophan at 0.1, 0.5, 1.0, 5.0 and 10 mM N and casein hydrolysate at 0.5, 1.0, 5.0 and 10 mM N were added to both Stage-I and Stage-II medium or only to Stage-II medium. The pH of both tryptophan and casein hydrolysate stock solutions was either not adjusted or adjusted to 5.7 in order to investigate the effect of pH on somatic embryogenesis.

The highest average number of somatic embryos produced per explant (33 embryos) and the highest total production of somatic embryos (132 embryos) were obtained from 5-9 mm explants growing on Stage-I and Stage-II medium supplemented with 0.1 mM N tryptophan which had been adjusted to pH 5.7 (Table 3.13). In general, adjusting the stock solutions to pH 5.7 caused the average number of somatic embryos produced per explant to be increased except for that obtained from explants growing on medium with 0.5 mM N casein hydrolysate.

Tryptophan at higher concentrations (5 mM N and 10 mM N) supplemented to Stage-I medium caused the explants to turn brown; although they recovered and produced callus following transfer to the Stage-II hormone-free medium, they were not able to produce somatic embryos. Unlike tryptophan, high concentrations of casein hydrolysate caused an excessive amount of friable callus to be produced but no somatic embryos were produced from this callus.

Table 3.11. The effect of tryptophan and casein hydrolysate added to both Stage-I and Stage-II MS medium on the production of somatic embryos from leaf lobes of cassava cultivar CMC 76

Nitrogen supplement (mM N)		Size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
Casein hydr.	Tryptophan				
0	0	1-3	20	4.0	16
		3-5	40	14.0	112
		5-9	10	23.0	46
0.5	0	1-3	20	7.5	30
		3-5	30	13.0	78
		5-9	10	1.0	22
1.0	0	1-3	0	0	0
		3-5	10	5.0	10
		5-9	10	6.0	12
5.0	0	1-3	10	7.0	14
		3-5	0	0	0
		5-9	0	0	0
0	0.1	1-3	0	0	0
		3-5	20	8.0	32
		5-9	20	33.0	132
0	0.5	1-3	30	5.5	33
		3-5	30	7.0	42
		5-9	0	0	0
0	1.0	1-3	0	0	0
		3-5	0	0	0
		5-9	10	5	10

Key :

Number of replicates : 25

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$PAR

Table 3.12. The effect of tryptophan and casein hydrolysate added to Stage-II MS medium on the production of somatic embryos from leaf lobes of cassava cultivar CMC 76

Concentration of nitrogen supplement (mM N)		Size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryo/ explant	total production of somatic embryos
Casein hydr.	Tryptophan				
0	0	1-3	20	4.0	16
		3-5	40	14.0	112
		5-9	10	23.0	46
0.5	0	1-3	0	0	0
		3-5	30	16.3	98
		5-9	0	0	0
1.0	0	1-3	10	1.0	2
		3-5	10	2.0	4
		5-9	0	0	0
0	0.1	1-3	60	4.8	57
		3-5	40	16.5	132
		5-9	0	0	0
0	0.5	1-3	0	0	0
		3-5	20	19.0	76
		5-9	10	9.0	18
0	1.0	1-3	0	0	0
		3-5	0	0	0
		5-9	10	2.0	4

Key:

Number of replicates : 25

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$PAR

Table 3.13. The effect of pH of tryptophan and casein hydrolysate stock solution on the production of somatic embryos from leaf lobes of cassava CMC 76

Concentration of nitrogen supplement (mM N)		pH	Size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
Casein hydr.	Tryptophan					
0.5	0	adjust.	1-3	20	7.5	30
			3-5	30	13.0	78
			5-9	10	1.0	2
		-	1-3	70	2.7	37
			3-5	60	4.0	48
			5-9	30	12.3	74
1.0	0	adjust.	1-3	0	0	0
			3-5	10	5.0	10
			5-9	10	6.0	12
		-	1-3	20	3.0	12
			3-5	0	0	0
			5-9	10	1.0	2
0	0.1	adjust.	1-3	0	0	0
			3-5	20	8.0	32
			5-9	20	33.0	132
		-	1-3	0	0	0
			3-5	30	7.0	42
			5-9	30	7.7	45
0	0.5	adjust.	1-3	30	5.7	34
			3-5	30	7.0	42
			5-9	0	0	0
		-	1-3	10	2.0	4
			3-5	10	4.0	8
			5-9	0	0	0

Key Number of replicates : 25

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$PAR

Of the concentrations of casein hydrolysate tested irrespective of the size of explants used, 0.5 mM N proved to be the best with respect to the frequency of explants producing somatic embryos and the average number of somatic embryos produced per explant. 3-5 mm leaf lobes, however, seemed to be the most responsive, if casein hydrolysate at this concentration was added to both Stage-I and Stage-II media (Table 3.11) or only to Stage-I medium (Table 3.12).

The type and concentration of amino acid and the stage of medium to which they were added also seemed to affect the frequency of normal plantlets derived from somatic embryo. This frequency could be increased from 3.4% to 15.8% by the addition of 0.1 mM N tryptophan, or to 18.2% by the addition of 1 mM N casein hydrolysate to both Stage-I and Stage-II medium (Table 3.11).

3.3.1.3.2.2. Effect of glutamine and proline on somatic embryogenesis of cassava cultivar CMC 76

50 mM N stock solution of both glutamine and proline were filter sterilised after their pH had been adjusted to 5.7 and they were then added either to both Stage-I and Stage-II medium or only to Stage-II medium. Since the availability of explants was limited, only one size of leaf lobes (3-5 mm) was used and these were incubated for 14 days in Stage-I medium and 21 days on Stage-II medium. Detailed results are shown in Table 3.14.

Proline seemed to have no beneficial effect on either the induction or the development of somatic embryos; adding proline in Stage-I medium (induction medium)

Table 3.14. The effect of proline and glutamine on the production of somatic embryos derived from leaf lobes of cassava cultivar CMC 76

Proline (mM N)		Glutamine (mM N)		% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
St-I	St-II	St-I	St-II			
-	-	-	-	10	1.0	2
50	50	-	-	0	0	0
-	50	-	-	10	1.0	2
-	-	50	50	10	1.0	2
-	-	-	50	20	5.0	20
50	50	50	50	0	0	0
-	50	-	50	20	2.0	8

Total χ^2 = 8.9, with 6 degrees of freedom, giving $P<0.05$

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

actually seemed to inhibit somatic embryogenesis. Similarly, a combination of proline and glutamine in the Stage-I medium resulted in no somatic embryos being produced

Adding 50 mM N glutamine to the Stage-II medium seemed to be beneficial, since the average number of somatic embryos produced per explant increased from one to five.

3.3.1.4. The effect of activated charcoal on somatic embryogenesis with cassava cultivar CMC 76

Owing to the restricted availability of explants from plants in the greenhouse, only one size of leaf lobes (3-5 mm) and one level of activated charcoal (5 gl^{-1}) - which was added either to Stage-I, together with 4 mgl^{-1} 2,4-D, or to Stage-II medium - were used. Tables 3.15 and 3.16 show the details of the results obtained.

Adding activated charcoal to the Stage-I medium seemed to result in lower frequency of explant producing somatic embryo (10%) than with the control (50%). Also, adding the activated charcoal to the Stage-II medium seemed to cause no further embryogenesis and the already induced somatic embryos seemed to grow faster and produce larger and darker cotyledons, although none could be converted to normal plantlets. It was also noted that most of the clumps of embryogenic tissues transferred to this medium produced roots instead of somatic embryos.

Table 3.15. The effect of activated charcoal added to Stage-I medium on somatic embryogenesis with cassava cultivar CMC 76

Level of activated charcoal (gl^{-1})	% explants producing somatic embryos	average number of somatic embryos produced per explant	total production of somatic embryos
0	50	22	220
5	10	10	20

Total $\chi^2 = 7.6^*$, with 1 degree of freedom, giving $P>0.05$

Table 3.16. The effect of activated charcoal addded to Stage-II medium on somatic embryogenesis with cassava cultivar CMC 76

level of activated charcoal (gl^{-1})	% clumps of somatic embryos possessing large cotyledons	% clumps of somatic embryos producing roots	% somatic embryos converting to normal plantlets
0	30	30	20
5.0	60	50	10

Total $\chi^2 = 3.6$, with 1 degree of freedom, giving $P<0.05$

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium and 21 days on hormone-free medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$PAR

3.3.1.5. Effect of high concentrations of sucrose on somatic embryogenesis with a range of South American cassava cultivars

The investigation was carried out with six South American (CIAT) cassava cultivars: CMC 40, CMC 76, MCol 22, MCol 113, MCol 216 and MCol 1684. Different concentrations of sucrose (2%, 8% and 14%, w/v), either alone or in combination with 4.0 or 10.0 mgl^{-1} 2,4-D were added to MS basal medium either for Stage-I alone or for both Stage-I and Stage-II to induce embryogenesis from three different ranges of size of leaf lobes (3-5, 5-7, and 7-9 mm). The explants were incubated for 3, 5, 7 or 14 days in Stage-I medium before they were transferred to Stage-II medium. The range of treatments is summarised in Table 3.17.

High concentrations of sucrose apparently did not give rise to better results in the induction of somatic embryogenesis. All explants growing on medium supplemented with 4.0 mgl^{-1} 2,4-D and 14% sucrose did not swell and they turned brown and dry by day 5. Increasing the concentration of 2,4-D to 10.0 mgl^{-1} made the effect of this level of sucrose even worse, except for 7-9 mm leaf lobes of MCol 1684, which produced structures similar to embryogenic tissues when they were transferred to medium with 4.0 mgl^{-1} 2,4-D and 2% sucrose. These tissues, however, were not able to produce somatic embryos, possibly as a result of the subsequent growth friable callus.

Sucrose at 8% in combination with 4.0 or 10.0 mgl^{-1} 2,4-D could only induce a morphogenic response when 7-9 mm leaf lobes of CMC 76 were used, although only foliose structure were formed following transfer to hormone-free medium.

Table 3.17. Summary of treatments used to investigate effect of high sucrose concentrations on somatic embryogenesis in cassava

Stage-I medium		Stage-II medium	
2,4-D concentration (mgl^{-1})	sucrose concentration (gl^{-1})	2,4-D concentration (mgl^{-1})	sucrose concentration (gl^{-1})
0	20	0	20
0	80	0	20
0	140	0	20
4.0	20	0	20
4.0	80	0	20
4.0	140	0	20
10.0	20	0	20
10.0	80	0	20
10.0	140	0	20
10.0	140	0	80
10.0	140	4.0	80

Key :

Number of replicates : 10

Basal medium : MS

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\ \mu Mm^{-2}s^{-1}$ PAR

Somatic embryos could only be obtained from 3-5 mm explants of CMC 76 and MCol 22, growing on standard medium which contained 2% sucrose supplemented with either 4.0 or 10.0 mgl^{-1} 2,4-D for 14 days before they were transferred to hormone-free medium. 3-5 mm leaf lobes of MCol 113 produced structures which were similar to embryogenic tissues when they were cultured on this medium for 5 days, but no somatic embryos were produced following tranfer to Stage-II medium.

3.3.1.6. The effect of the age of clonal plants on somatic embryogenesis in leaf lobes of cultivar CMC 76

Since different sizes of leaf lobes have given different embryogenic responses (see Section 3.3.1.1) it was decided to investigate further the influence of the parental plants on subsequent embryogenic behaviour under *in vitro* conditions. Young leaf lobes (1-3, 3-5, and 5-7 mm in size) were therefore taken from clonal plants growing in the greenhouse for either two or four months after the plants had been cut back. The medium used, period of incubation and culture conditions were as described in previous experiments (Section 3.3.2.1). Table 3.18 shows the frequency of explants producing somatic embryos, the average number of somatic embryos produced per explant and the total production of somatic embryos.

Explants taken from 4 month old clonal plants in the greenhouse produced somatic embryos at a higher frequency than those taken from 2 months old plants, irrespective of the size of leaf lobes used. The larger size, however, led to a higher frequency of

Table 3.18. Effect of the age of clonal plants on embryogenesis in leaf lobes of cultivar CMC 76.

Age of clonal plants (months)	size of leaf lobes (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
2	1-3	20	1	2
	3-5	40	2	8
	5-7	50	5	25
4	1-3	40	8	32
	3-5	70	20	140
	5-7	80	26	208

Table 3.19. The effect of concentration of 2,4-D and duration of the Stage-I treatment on somatic embryogenesis of cassava leaf lobes cultured in liquid medium

level of 2,4-D (mgl^{-1})	duration of Stage I (days)	% explants producing somatic embryos	average number of somatic embryos/ explant	total production of somatic embryos
2.0	13	30	3.7	22
	36	10	3.0	6
4.0	13	50	4.3	43
	36	20	20.5	82
6.0	13	30	3.7	22
	36	40	14.3	114

Key : Number of replicates : 20

Basal medium : MS supplemented with 2% (w/v) sucrose

Regeneration medium : hormone-free MS medium

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod,

$30 \mu Mm^{-2}s^{-1}$ PAR

embryogenesis (up to 80%) and a higher average number of somatic embryos produced per explant (up to 26).

3.3.2. Induction of somatic embryogenesis in stationary liquid media with cassava cultivar CMC 76

As a comparison with semi-solid medium, stationary liquid medium supplemented with a range of 2,4-D concentrations (2.0-6.0 mgl^{-1}) including including that (4.0 mgl^{-1}) which was proved to be optimal with semi-solid medium was tested for its effect on the induction of somatic embryogenesis. Explants were either floated on the medium, placed on different types of filter-paper bridges, or on sloped filter papers in 5 cm plastic Petri dishes containing different volume of medium. In addition, explants were also placed on a filter-paper bridge in glass test tubes containing different volume of medium. In order to investigate the effect of gas circulation, after covering test tubes with aluminium caps, they were either sealed with parafilm or left unsealed. Due to the limited availability of explants, only one size of (3-5 mm) leaf lobes was used. Unless otherwise stated, all explants were subjected to a two-stage culture procedure involving transfer to the Stage-II medium after 14 days. The frequency of explants producing somatic embryos and the number of embryos produced per explant were scored.

3.3.2.1. Effect of concentration of 2,4-D and duration of the Stage-I treatment on somatic embryogenesis of cassava leaf lobes cultured in liquid medium

Leaf lobes (3-5 mm in size) were cultured floating on Murashige and Skoog liquid medium supplemented with either 2.0, 4.0 or 6.0 mgl^{-1} 2,4-D in 5 cm plastic Petri dishes for either 13 or 36 days before they were transferred to Stage-II medium. Table 3.19 shows the frequency of explants producing somatic embryo and the average number of somatic embryos produced per explant.

The duration of incubation proved to be important in determining the average number of somatic embryos produced per explant and the highest average value (20.5) was obtained with explants incubated for 36 days in the Stage-I medium containing 4.0 mgl^{-1} 2,4-D, and the highest total production of somatic embryos (114) was obtained from medium with 6.0 mgl^{-1} 2,4-D. Although 13 days of incubation in Stage-I medium brought about the highest frequency of explant producing somatic embryo (50%) when the explants were cultured in medium containing 4.0 mgl-1 2,4-D, the total somatic embryos produced was lower than those incubated for 36 days because the average number of somatic embryos produced per explant was low. Plate 3.2A shows the induction of somatic embryogenesis from leaf lobes of cassava cultured floating on liquid medium.

3.3.2.2. Effect of the volume of liquid medium on somatic embryogenesis of leaf lobes of cassava cultivar CMC 76

The 3-5 mm leaf lobes were floated in different volume of media (3.0, 5.0 and 7.0 ml) in 5.0 cm Petri dishes. As a comparison with those cultured in 7.0 ml medium,

Table 3.20. Effect of the volume of liquid medium on somatic embryogenesis of floated leaf lobes of cassava cultivar CMC 76

Method of culture	Volume of medium (mm)	% explants producing somatic embryos	average number of somatic embryos/ explant	categories of somatic embryos based on size (%)		
				large (5-9 mm)	medium (1-5 mm)	small (<1 mm)
Floated	3.0	30	2.3	0	0	100.0
	5.0	20	7.5	13.3	26.7	60.0
	7.0	50	7.8	30.9	60.0	30.9
on sloped filter paper	7.0	50	6.0	43.3	16.7	40.0

Total χ^2 = 2.1, with 2 degrees of freedom, giving $P<0.05$

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Regeneration medium : hormone-free MS medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\mu Mm^{-2}s^{-1}PAR$

Table 3.21. Effect of the width and the number of layers of filter paper on somatic embryogenesis with leaf lobes of cassava cultivar CMC 76

Number of layers of filter paper	width of filter paper bridge (mm)	% dead explants	% explants producing callus	% explants producing somatic embryos
1	9	20	30	0
	18	80	10	0
2	9	20	80	10
	18	40	20	0

Total χ^2 = 12.7*, with 3 degrees of freedom, giving P>0.05

Table 3.22. Effect of the type of containers and aeration on somatic embryogenesis with leaf lobes of cassava cultivar CMC 76 cultured in liquid medium

Type of container	% explants producing somatic embryos	average number of somatic embryos produced per explant	total production of somatic embryos
Test tubes			
* sealed	30	5.3	16
* unsealed	20	3.0	6
Petri dishes	40	6.0	24

Total χ^2 = 0.9, with 2 degrees of freedom, giving P<0.05

Key :

Number of replicates : 10

Basal medium : MS supplemented with 4.0 mgl^{-1} 2,4-D and 2% (w/v) sucrose

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

explants were also placed on sloped filter papers in Petri dishes containing that amount of medium. Table 3.20 shows the frequency of explants producing somatic embryos, the average number of somatic embryos produced per explant and the proportions of the different sizes of somatic embryos produced.

From the volumes of medium tested, 7.0 ml gave rise to the highest frequency of explants producing somatic embryos (50%) and the highest average number of somatic embryos produced per explant (7.8 embryos). This treatment also resulted in the highest frequency of medium-sized somatic embryos (60%) which was the most appropriate size to regenerate to normal plantlets. The number of somatic embryos produced per explant would seem to increase with the volume of medium up to 7.0 ml. With this volume of medium there was no difference between those leaf lobes which were floated and those placed on sloped filter paper in terms of the frequency of explants producing somatic embryos (Table 3.20).

3.3.2.3. Effect of the width and the number of layers of filter paper on somatic embryogenesis with leaf lobes of cassava cultivar CMC 76

This experiment was intended to be a comparison between the response of leaf lobes floated in direct contact with the medium, and those placed on sloped filter paper (almost contact with the medium) and those placed on filter bridges (indirect contact with the medium). The size of Petri dishes used and the volume of the medium were the same as used in Section 2.3.2.1. One or two layers of either 9 mm x 85 mm or 18 mm x 85 mm filter papers (Whatman No.3) were folded to an inverted "U", to form the bridges for the support of the explants.

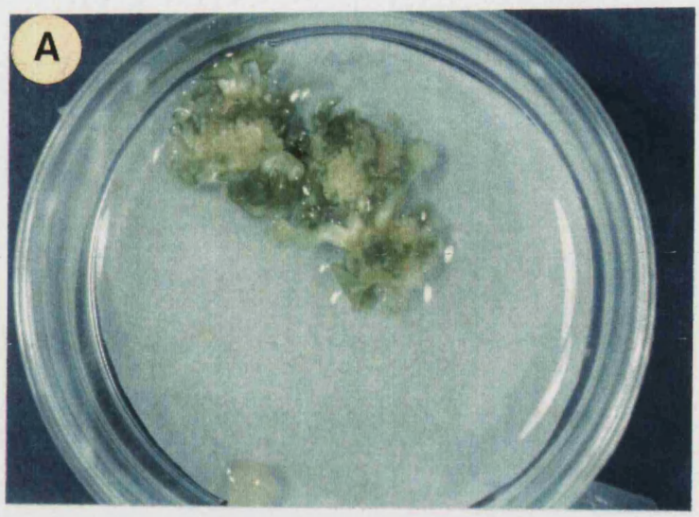
Plate 3.2.

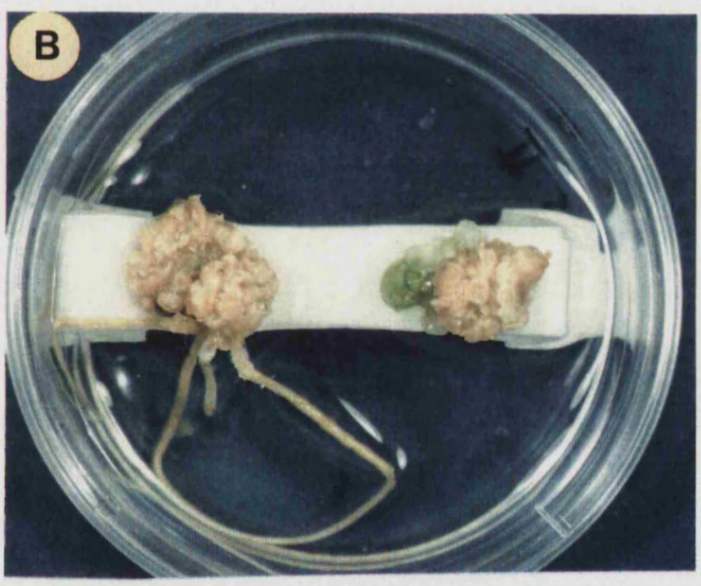
Induction of somatic embryos from immature leaf lobes of cassava cultivar CMC 76 in MS liquid medium

A. Induction of somatic embryogenesis from leaf lobes floating on MS liquid medium supplemented with 6.0 mgl^{-1} 2,4-D in Petri dishes (see Section 3.3.2.1). x4.5.

B. Induction of somatic embryogenesis from leaf lobes placed on a filter-paper bridge in Petri dishes containing 4.0 mgl^{-1} 2,4-D (see Section 3.3.2.3). x4.5.

C. Induction of somatic embryogenesis from leaf lobes placed on a filter-paper bridge in test tubes containing different volumes of liquid medium (3.0, 5.0 or 7.0 ml) supplemented with 4.0 mgl-1 2,4-D (see Section 3.3.2.4). x4.5.


A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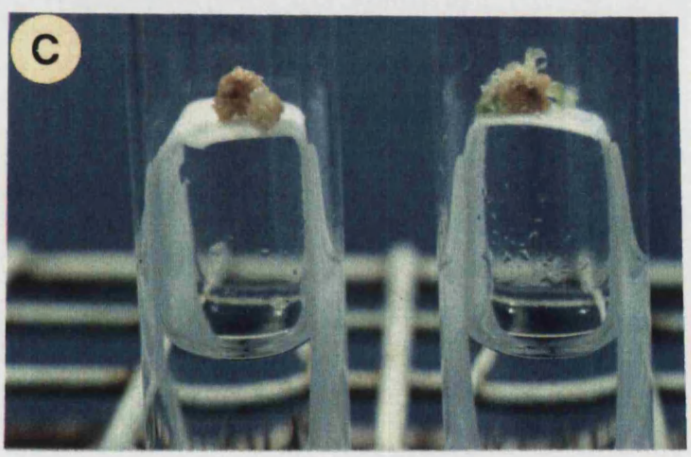

C

Somatic embryos were only produced from explants growing on liquid medium supported with two layers of filter papers 9 mm x 85 mm in size. Two layers of filter bridges of both sizes seemed to give better results than one layer in terms of callus production and somatic embryogenesis Plate 3.2B). Most of the explants (80%) supported on one layer of filter paper in the form of a bridge turned brown and dry (Table 3.21).

3.3.2.4. Effect of the type of containers and aeration on somatic embryogenesis with leaf lobes of cassava cultivar CMC 76 cultured in liquid medium

A comparison was made between the effect of culturing explants in glass test tubes (24 mm x 150 mm) and those cultured in plastic Petri dishes (5.0 cm). The same volume of medium (7.0 ml) was used in both types of container with the explants supported on filter paper bridges (see Section 2.3.2.3). The test tubes were either sealed with parafilm or left unsealed in order to determine the effect of aeration on somatic embryogenesis, while all Petri dishes were sealed.

The explants cultured in the Petri dishes gave better results than those cultured in both the sealed and the unsealed glass test tubes in terms of the frequency of explants producing somatic embryos and the average number of somatic embryos produced per explant (Table 3.22). Culturing the explants in sealed test tubes (Plate 3.2C) caused the frequency of explants producing somatic embryos to be reduced slightly 40% to 30% and the average number of somatic embryos produced per explant to be reduced from 6 to 5.3 embryos. The frequency and number of somatic embryos per explant decreased further to 20% and 3 embryos respectively, if the tubes were left unsealed. Keeping the test tubes unsealed also caused the growth of somatic embryos to be very slow with the result that all somatic embryos produced in this type of container were smaller than those produced in sealed test tubes or in sealed Petri dishes.

3.4. DISCUSSION

The results (Section 3.3.1.1.1 and Section 3.3.1.1.2) showed that somatic embryogenesis in cassava is strongly influenced by genotype and that the genotypes responded differently to the growth regulators 2,4-D, picloram and dicamba. Some of the cultivars which were recalcitrant with 2,4-D, responded to either picloram or dicamba but the required concentrations of these growth regulators were considerably higher than those used for inducing somatic embryogenesis in *Glycine sp.* (Gamborg *et.al.* ,1983), *Pisum sativum* (Jacobsen and Kysely, 1984), *Musa sp.* (Escalant and Teisson, 1989) and *Dactylis glomerata* (Gray and Conger, 1985).

Both the concentration of 2,4-D and the period of incubation seem to be very important in relation to the induction of somatic embryogenesis of cassava, and it was shown (Section 3.3.1.2.1 and Section 3.3.1.2.2) that a combination of 4.0 mgl^{-1} 2,4-D with 13 days for Stage I incubation seem to be optimal for obtaining the highest frequency of explants producing somatic embryos (60%). These results would seem to be at variance with those of Stamp and Henshaw (1982) in which 8.0 mgl^{-1} 2,4-D and an incubation period of 27 or 31 days were shown to be optimal. This, however, was probably due to differences in the sizes of leaf lobes used, since it was shown in the present work (Section 3.3.1.1.1) that the choice of size was very important.

In addition to the size of explant, the type of explant plays an important role with regard to the induction of somatic embryogenesis in cassava. Except for 3-5 mm leaf lobes, axillary buds and meristems from axillary buds, other parts of plants tested (leaf sections, petioles, stem sections and roots) failed to undergo embryogenesis (Section 3.3.1.1.2). Vasil (1982) demonstrated that somatic embryogenesis of some cereals was localized in the juvenile region.

Another factor which affected cassava somatic embryogenesis was the type of medium used. The superiority of Murashige and Skoog medium to B5 medium, was possibly because of the difference in the ratio of nitrate to ammonium (20:40 and 25:2 respectively). Reinert *et.al.* (1967); Tazawa and Reinert (1969) concluded that supplementation of nitrogen was necessary if White's medium was used. They also showed that there was an interaction between 2,4-D and the concentration of nitrogen which affected embryo development, so that, additional nitrogen was required to allow embryos to develop if White's medium supplemented with 2 x 10 M 2,4-D was used. The importance of the nitrate : ammonium ratio for the induction and regeneration of somatic embryos has also been reported by Halperin and Wetherell (1965); Reinert et.al. (1967) and Meijer and Brown (1987). The results of Reinert *et. al.* (1967), however, were contradictory in that it was the concentration and not the form of the nitrogen in medium which was important; while the results from Section 3.3.1.3.1 indicated that both concentration and the form of nitrogen would seem to be important. In the case of cassava, nitrate seemed to be more important than ammonium (Section 3.3.1.3.1) with regard to the production of somatic embryos and of normal plantlets which was in contrast to the work of Halperin and Wetherell (1965) and Meijer and Brown (1987). These authors also emphazised that ammonium was required for the differentiation of the somatic embryos.

Exogenous amino acids, except tryptophan, is possibly not beneficial for the induction of somatic embryos (Section 3.3.1.3.2.1) The positive effect of tryptophan (Section 3.3.1.3.2.1), according to Siriwardana and Nabors (1983),in work with rice, was thought to be a result of its role as IAA precursor. The failure of proline in inducing somatic embryos is presumably because the dependence of the process upon ammonium ion (Stuart and Strickland, 1984). They reported that in the presence of

proline, embryo numbers can be increased by 40% in SH medium as a result of the optimization of the concentration of ammonium ions and proline simultaneously. In addition, they also showed that glutamine was the only amino acid capable of stimulating somatic embryogenesis with *Medicago sativa* L. which was not dependent on ammonium.

Casein hydrolysate added to both Stage-I and Stage-II medium would seem to be important in aiding the differentiation of cassava somatic embryos to obtain a higher frequency of normal plantlets (Section 3.3.1.3.2.1). These results support those of Banerjee and Gupta (1976) in work with *Nigella sativa* and Meijer and Brown (1987) in work with *Medicago sativa* in that the addition of casein hydrolysate instead of coconut milk to the medium improved the differentiation of somatic embryos ultimately the number of embryos which grew into normal plantlets. In carrot, casein hydrolysate supported both induction and differentiation of somatic embryos (Wetherell and Dougall, 1976).

Adjusting the pH of the amino acid stock solutions would seem to be crucial since a higher frequency of embryogenesis was obtained (Section 3.3.1.3.2.1). The original pH of tryptophan solution which was 10.4 would not seem to be favorable for embryogenesis which requires the pH between 5.7 to 7.0 (Lazzeri *et.al.*, 1987). A high medium pH, according to Skirvin *et.al.* (1986) and Lazzeri *et.al.*, (1987) reduced normal embryo production.

When activated charcoal at 5.0 gl^{-1} was added to the Stage-I medium most of the leaf lobes that were cultured remained green without forming callus or somatic embryos (Section 3.3.1.4). With this concentration of activated charcoal, it is likely that all of the 2,4-D, which had been added at 4.0 mg^{-1}, is adsorbed (Weatherhead *et al*, 1979).

Nwankwo and Krikorian (1986) in work with oil palm emphazised the need to increase the level of auxin to 10-70 mgl^{-1} when activated charcoal at 0.5 gl^{-1} was used. The results here (Section 3.3.1.4), however showed that the addition of activated charcoal enhanced the growth and development of the somatic embryos, which is in agreement with the work of Fridborg and Eriksson (1975) with carrot, Tisserat and De Mason (1980) with date palm, and Litz and Conover (1980) with *Carica stipulata.*

In cassava, high concentrations of sucrose would not seem to be beneficial for the induction of embryogenesis even with the so called recalcitrant cultivars (Section 3.3.1.5). This result in agreement with the results obtained by Meijer and Brown (1987) in work with *Medicago sativa*, showed that high and low sucrose concentrations inhibited somatic embryogenesis so that there was no reason to deviate from the standard sucrose level (2-3%). In contrast, Lu *et.al.* (1982) and Conger *et.al.* (1987) in work with *Zea mays*, Chandler and Beard (1983) and Finer (1987) in work with sunflower showed that somatic embryos were induced by placing explants on a medium containing high sucrose (6-12%, w/v) and 2,4-D.

The abnormal forms of somatic embryos such as single, multiple, fused and curled cotyledons could possibly be due to external factors, such as the ratio of nitrate to ammonium (Section 3.3.1.3.1) and concentration of growth regulators, affecting such internal factors as cell division. Ammirato (1983) argued that poor development of cotyledons was a result of premature vacuolation and inadequate cell division. Changes in cell division, enlargement and differentiation are also believed to affect cotyledons formation. In normal cotyledon formation, the proembryo undergoes a series of cell divisions and a ring of cells forms a cotyledonary collar. If the size of cotyledonary ring is altered by too many cell divisions and, or premature cell enlargement, then more than two centres can initiate to form multiple cotyledons. The fused or faciated cotyledons

will be obtained as a result of the discontinuity of cell divisions in the cotyledonary ring (Ammirato, 1983). Tisserat *et.al.* (1979), however, state that the causes of abnormalities which are a common phenomenon in somatic embryogenesis, are not well understood. Ammirato and Steward (1981) suggested that imbalances in cultural medium or environment might be involved, while Trigiano *et.al.* (1988) suggested that a non-functional meristem or the requirement for a more complex regime of growth regulators and media might be responsible for the failure of *Cercis canadensis* somatic embryos to form shoots.

The type of culture vessels and gas aeration would also seem to be important in somatic embryogenesis of cassava (Section 3.3.2.4). This, is in agreement with Ammirato's (1982) results with caraway somatic embryos which showed a considerable difference in the degree of abnormality of embryos obtained from cells grown in Erlenmeyer flasks and from cells grown in more gently rotated tumble tubes. It was also possible that the gaseous environment might have played an important role in the different response of cassava explants grown with different volumes of medium (Section 3.3.2.2) or in Petri dishes and in test tubes (Section 3.3.2.4). Zig and Gadasi (1986) concluded that the exposure of the somatic embryos to the gas phase induced the dedifferentiation so that they required continuous bathing in the liquid medium in order to remain differentiated. Dunwell (1979) believed that the composition of gas mixture in culture vessels had a great influence on the number of embryo produced in anther cultures of *Nicotiana tabacum.*

CHAPTER 4

LONG-TERM MAINTENANCE OF EMBRYOGENIC COMPETENCE

4.1. INTRODUCTION

For the following reasons, plant regeneration is a rate-limiting step in genetic manipulation programmes involving cassava : the influence of genotype on morphogenetic competence is strong, morphogenetically competent tissues appear to be very localized in the plant and competence declines as disorganization increases in culture systems, with the result that rapidly growing non-competent cells and tissues can eventually dominate cultures. It is desirable, therefore, that the growth of the embryogenic tissues should be maintained over as long a period as possible.

Callus and suspension cultures which are initially capable of embryogenesis often show a progressive decline and sometimes a complete loss of this morphogenic ability as they are maintained in cultures through repeated subcultures (Fridborg and Eriksson, 1975); Negrutiu and Jacobs, 1978). To explain the loss of morphogenic potential, Bhojwani and Razdan (1983) have proposed three hypotheses based, respectively, on genetic, physiological and competitive mechanisms.

The genetic hypothesis proposes that the loss of morphologenic potential is a result of irreversible genetic changes taking place in the cultures. It is well known that such changes, including both chromosomal and point mutation occur (Krikorian *et al.*, 1981) but it is not easy to demonstrate that they are actually responsible for the loss of competence.

Physiological explanations include the possibility that an altered hormonal balance within the cells or tissues, or sensitivity of the cells to exogenous growth substances may cause the decline of morphogenic potential (Fridborg and Eriksson, 1975; Negrutiu and Jacobs, 1978).

Competition between populations of competent and non-competent cells derived from cells in the original explant, could also lead to an overall loss of competence if the culture conditions tend to favour the latter.

With all of these mechanisms, it is possible that suitable manipulation of the culture conditions might slow down or even prevent the loss of competence. This has been attempted by the use of cold treatment (Syono, 1965), the addition of charcoal (Fridborg and Eriksson, 1975) or the application of ABA (Qureshi, 1989). Reinert *et al.*(1968, 1970) could reinduce embryogenesis in cultures which had ceased to form embryos simply by withdrawal of the 2,4-D of the medium.

Several factors seem to play an important role in the maintenance of embryogenic tissues such as growth regulator and subculture regimes. Medium supplemented with 2,4-D alone has been used to maintain embryogenic carrot cultures (Reinert *et al.*, 1968, 1970), *Carica papaya* cultures (Litz and Conover, 1983) and sugarcane cultures (Chen *et al.*, 1988). Other embryogenic cultures of plant species such as *Albizia richardiama* (Tomar and Gupta, 1988) was maintained on medium containing BAP only, whereas embryogenic cultures of cucumber (Bergervoet, 1989) and of rice (Ram and Nabors, 1984) were maintained in medium with a combination of 2,4-D and cytokinin. In addition to growth regulators, the subculture regime proved to be critical in the success of maintaining cultures of plant species such as *Zea diploperennis* (Swedlund and Locy, 1988).

Exogenous abscisic acid has been reported to show a positive effect in maintaining and enhancing embryogenic callus formation from monocotyledonous explants (Rajasekaran *et al.*, 1987; Reddy and Reddy, 1987; Vasil and Vasil, 1981). Ammirato

(1977); Kamada and Harada (1981) reported that at low concentrations (10^{-6} to 10^{-7} M) ABA helped with the normalization of embryogenesis in carraway cultures; while Kochba *et al.*(1978); Fujimura and Komamine (1975) demonstrated that a short treatment with ABA increased somatic embryogenesis in *Citrus sinensis* and the formation of early-stage carrot somatic embryos respectively.

In principle, there are two possible approaches to the problem of maintaining embryogenic cultures on a long-term basis : factors favouring the stable proliferation of the semi-organized embryogenic callus rather than the disorganized non-competent callus may be optimized or, alternatively, a system based on prolonged secondary embryogenesis might be devised. In this investigation, both approaches were used.

4.2. RESULTS

4.2.1. The induction of secondary embryogenesis from primary somatic embryos

In order to prolong the proliferation of somatic embryos at a high frequency, factors possibly influencing secondary embryogenesis were investigated, including the size and the colour (as an indicator of the stage of development) of primary embryos, the concentration of 2,4-D, in both liquid and semi-solid medium. Individual or clumps of somatic embryos used were those had been cultured on medium supplemented with 2.0 mgl^{-1} or 4.0 mgl^{-1} 2,4-D under conditions as mentioned in Chapter 2. The frequency of secondary embryogenesis was scored at day 30.

4.2.1.1. The effect of the size of somatic embryos, concentration of 2,4-D in both liquid and semi-solid medium on the induction of embryogenesis

Two different size ranges of primary somatic embryos (1.5-3.5 and 3.5-5.5 mm) and three different concentration of 2,4-D (2.0, 4.0, 6.0 mgl^{-1}) were added to both liquid and semi solid medium.

The highest frequency of secondary embryogenesis (100%) was obtained by culturing 1.5-3.5 mm primary embryos on either semi-solid or liquid medium supplemented with 4.0 mgl^{-1} 2,4-D. It seemed that 4.0 mgl^{-1} 2,4-D was the optimum concentration for this size range since increasing the concentration to 6.0 mgl^{-1} decreased the frequency to 30-40% (Table 4.1). On the other hand, the frequency of 3.5-5.5 mm primary embryos producing secondary embryos continued to increase as the

Table 4.1. The effect of the size of somatic embryos, concentration of 2,4-D and state of medium on the production of somatic embryos

State of medium	level of 2,4-D (mgl^{-1})	size of primary embryos (mm)	% primary embryos producing secondary embryos
semi-solid	2.0	1.5-3.5	90
		3.5-5.5	10
	4.0	1.5-3.5	100
		3.5-5.5	20
	6.0	1.5-3.5	40
		3.5-5.5	60
liquid	2.0	1.5-3.5	80
		3.5-5.5	50
	4.0	1.5-3.5	100
		3.5-5.5	60
	6.0	1.5-3.5	30
		3.5-5.5	100

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\,\mu Mm^{-2}s^{-1}$ PAR

concentration of 2,4-D increased up to 6.0 mgl^{-1}, particularly in liquid medium, but the actual number of embryos produced was always lower.

4.2.1.2. The effect of the size and colour of somatic embryos, and the concentration of 2,4-D on the secondary embryogenesis

Since primary somatic embryos of the same size could be different in terms of colour or maturity, they were separated into two groups with different colours (light and dark green). Light and dark green embryos from different size ranges (1.5-3.5 and 3.5- 5.5 mm) were cultured on semi-solid medium supplemented with either 2.0 or 4.0 mgl^{-1} 2,4-D. Table 4.2. shows the frequency of secondary embryogenesis of primary embryos at different stage of development.

Observations revealed that size and colour of somatic embryos were interdependent in determining the frequency of secondary embryogenesis. Light green primary embryos in the size range 1.5-3.5 mm cultured on 4.0 mgl^{-1} 2,4-D medium gave rise to the highest frequency of secondary embryogenesis (100%). Plates 4.1A-C show the formation of secondary somatic embryos on the cotyledons of primary embryos.

At each 2,4-D concentration tested , there seemed to be a regular pattern of response in which frequency of secondary embryogenesis decreased as the size and the intensity of colour of primary embryos increased. Medium with 4.0 mgl^{-1} 2,4-D, however, produced a higher frequency of response than medium with 2.0 mgl^{-1} 2,4-D.

Table 4.2. Effect of the size and colour of somatic embryos and the concentration of 2,4-D on the production of somatic embryos

level of 2,4-D (mgl^{-1})	primary somatic embryos		% primary somatic embryos producing secondary embryos
	size (mm)	colour	
2.0	1.5-3.5	light green	80
		dark green	40
	3.5-5.5	light green	20
		dark green	0
4.0	1.5-3.5	light green	100
		dark green	80
	3.5-5.5	light green	60
		dark green	30

Total overall size $\chi^2 = 17.1^{**}$, with 1 degree of freedom, giving $P>0.01$
Total overall colour $\chi^2 = 5.5^{*}$, with 1 degree of freedom, giving $P>0.05$

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod,

$30 \mu Mm^{-2}s^{-1}$ PAR

Plate 4.1.

The induction of secondary somatic embryos from primary somatic embryos of cassava cultivar CMC 76 on medium supplemented with 4.0 mgl^{-1} 2,4-D.

A. The induction of embryogenic tissues from individual primary somatic embryos (ct = cotyledons of primary embryo, set = secondary embryogenic tissues). x40.5.

B. Further growth of secondary somatic embryos on the surface of the cotyledons of primary somatic embryos (ct = cotyledons of primary somatic embryo, set = secondary embryogenic tissues). x40.5.

C. Later stage of secondary somatic embryos on the surface of the cotyledons of primary somatic embryos (ct = cotyledons of primary somatic embryo, set = secondary embryogenic tissues). x40.5.

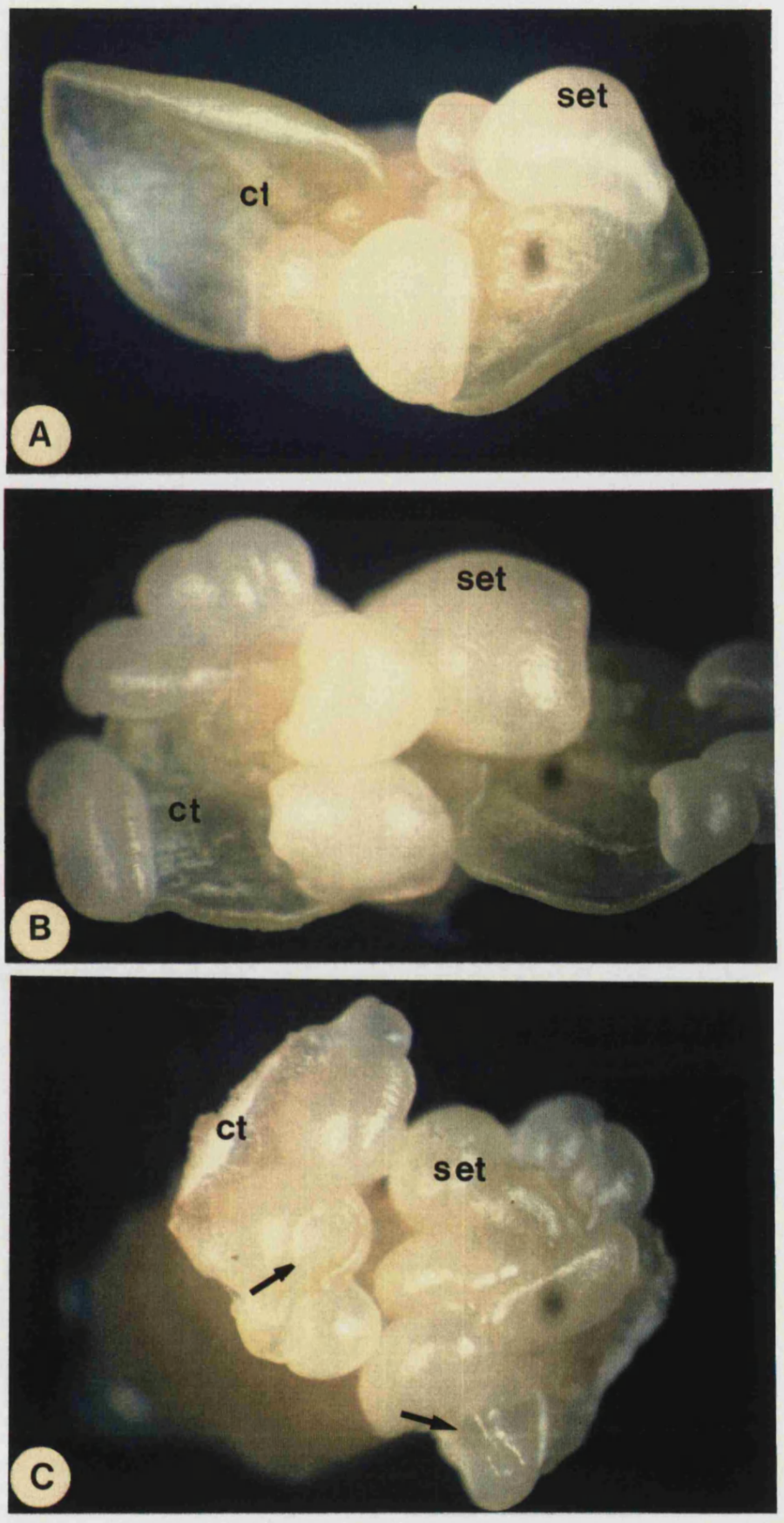

set
ct
A
set
ct
B
ct
set
C

Plate 4.2.

The proliferation of secondary somatic embryos on medium containing 4.0 mgl^{-1} 2,4-D.

A. The proliferation by budding of somatic embryos from older secondary somatic embryos. x40.5

B. Extensive proliferation of secondary somatic embryos. x40.5.


A

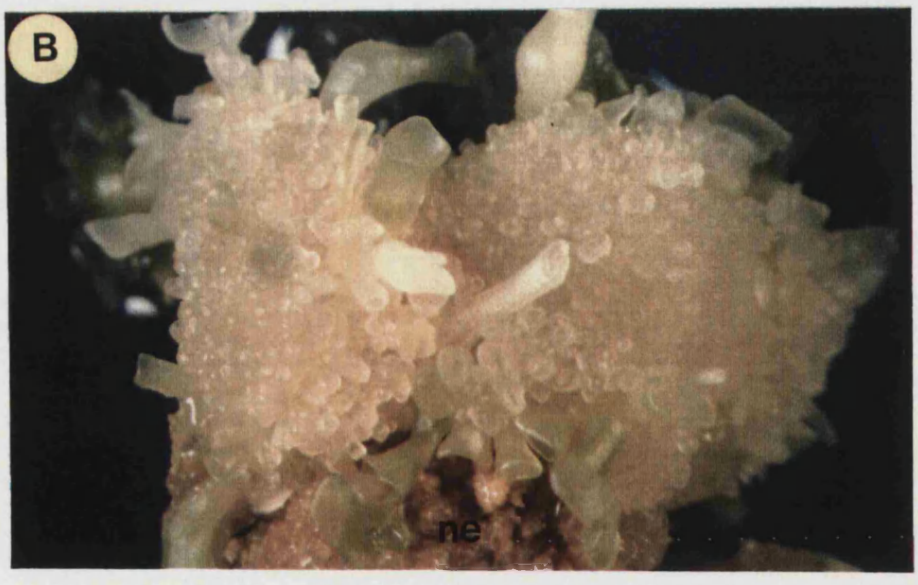

B
ne

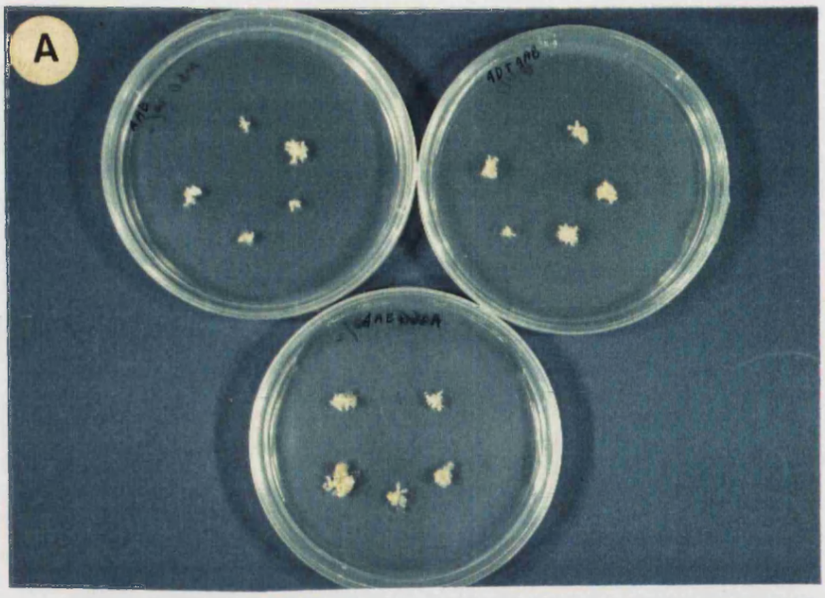
Plate 4.3.

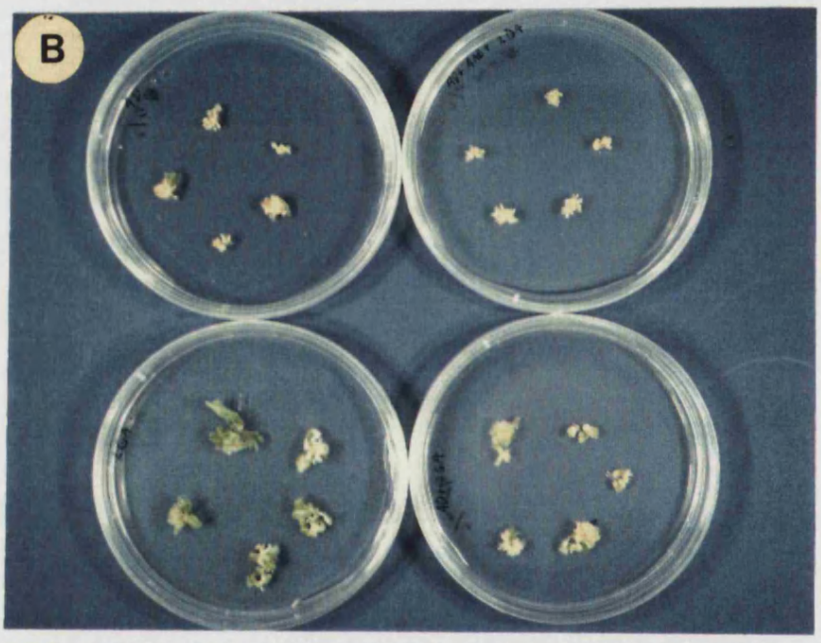
Maintenance of clumps of somatic embryos on medium supplemented with either ABA, GA_3 or activated charcoal either alone or in combination with 2,4-D.

A. Regeneration of clumps of somatic embryos after having been maintained on a medium supplemented with 2.0 mgl^{-1} GA_3 alone, 4.0 mgl^{-1} 2,4-D alone, a combination of 4.0 mgl^{-1} 2,4-D and 4.0 mgl^{-1} ABA and 2.0 mgl^{-1} GA_3, or a combination of 4.0 mgl^{-1} 2,4-D and 2.0 mgl^{-1} GA_3 (clock-wise). x 4.5.

B. Regeneration of clumps of somatic embryos after having been maintained on a medium supplemented with either 4.0 mgl^{-1} 2,4-D alone, or a combination of 4.0 mgl^{-1} 2,4-D and 4.0 mgl^{-1} ABA, or a combination of 4.0 mgl^{-1} ABA and 2.0 mgl^{-1} GA_3 for 40 days (clock-wise). x4.5.

C. Regeneration of clumps of somatic embryos on hormone-free medium with or without charcoal after having been maintained on a medium with 2,4-D alone or a combination of 2,4-D and activated charcoal. x4.5.


A


B


C

In general, therefore, since the light green embryos were superior to the darker green embryos in terms of their ability to undergo secondary embryogenesis, the colour of embryos is a useful indicator of the propensity for secondary embryo production.

4.2.2. The effect of ABA, GA_3, and activated charcoal on the maintenance of embryogenic competence

Single somatic embryos or embryogenic clumps were cultured on medium containing gibberellic acid at 2.0 mgl^{-1}, or activated charcoal at 1.0 gl^{-1}, or ABA at three different concentrations (0.2, 2.0 and 4.0 mgl^{-1}), either alone or in combination with 4.0 mgl^{-1} 2,4-D. The results are shown in Table 4.3 and Plates 4.3A-C.

ABA or GA_3 used alone seemed to encourage the growth of the somatic embryos as indicated by the enlargement of their cotyledons. On the other hand, ABA at 0.2-2.0 mgl^{-1} and GA_3 (2.0 mgl^{-1}) in combination with 2,4-D (4.0 mgl^{-1}) prevented the germination of somatic embryos without causing the loss of embryogenic competence. With these combinations of growth regulators the new embryos appeared to be more uniform, shiny in appearance and circular in shape. ABA at 4.0 mgl^{-1}, however, seemed to be too high for somatic embryos, particularly for those cultured individually, since further embryogenesis was inhibited. The clumps of somatic embryos became water logged with the result that they lost their embryogenic competence if they were left on this medium. Individual somatic embryos seemed to be more sensitive to ABA than clumps of somatic embryos, since they turned white and lost their embryogenic competence on medium with 2.0 mgl^{-1} ABA (Table 4.3).

Table 4.3. The effect of ABA and GA_3 alone or in combination with 2,4-D on the production of somatic embryos

Nature of somatic embryos	Media				%explants showing white appearance	%explants producing secondary embryos
	2.4-D (mgl^{-1})	ABA (mgl^{-1})	GA^3 (mgl^{-1})	activated charcoal (gl^{-1})		
individuals	-	-	-	-	40	10
	-	-	-	5.0	10	0
	-	-	2.0	-	10	0
	-	0.2	-	-	70	0
	-	2.0	-	-	100	0
	-	4.0	-	-	50	0
	-	4.0	2.0	-	30	20
	4.0	-	-	-	0	70
	4.0	-	-	5.0	0	10
	4.0	-	2.0	-	0	30
	4.0	0.2	-	-	20	30
	4.0	2.0	-	-	70	0
	4.0	4.0	-	-	60	40
	4.0	4.0	2.0	-	50	20
clumps	-	-	-	-	40	10
	-	-	-	5.0	70	0
	-	-	2.0	-	0	0
	-	0.2	-	-	100	0
	-	2.0	-	-	100	0
	-	4.0	-	-	70	0
	-	4.0	2.0	-	50	20
	4.0	-	-	-	0	90
	4.0	-	-	5.0	30	30
	4.0	-	2.0	-	0	80
	4.0	0.2	-	-	20	80
	4.0	2.0	-	-	30	70
	4.0	4.0	-	-	50	60
	4.0	4.0	2.0	-	20	70

Key: Number of replicates : 10

Basal medium : MS supplemented with 2% sucrose

Size of fraction : < 0.5 mm (Fraction III)

0.5-1.5 mm (Fraction II)

> 1.5 mm (Fraction I)

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod,

$30 \mu Mm^{-2}s^{-1}$ PAR

Activated charcoal weakened the effect of the 2,4-D so that only 10% of individual embryos and 30% of clumps of embryos could undergo further embryogenesis, in comparison with 70% and 90%, respectively, when 2,4-D was used alone. The presence of activated charcoal also affected the development of somatic embryos, with the result that the growth of cotyledons was accelerated and rooting was inhibited (Plate 4.3C).

4.2.3. The effect of sucrose on the maintenance of embryogenic competence

The effect of three different concentration of sucrose (2.0, 4.0 and 6.0%), either alone or in combination with 2,4-D on the maintenance of both individual and clumps of somatic embryos were investigated. Table 3.4 shows the effect of sucrose on the frequency of embryos losing the embryogenic competence and the frequency of secondary embryogenesis.

Apparently, only standard medium (supplemented with 2.0% sucrose and 4.0 mgl^{-1} 2,4-D) could be used to maintain the embryogenic tissues, since the higher sucrose concentrations (4.0% alone or 6.0% either alone or in combination with 2,4-D) caused the majority of clumps of somatic embryos (60-90%) to turn white indicating a loss of embryogenic competence which could not be restored by subsequent transfer to regeneration medium. The negative effect of the higher sucrose concentration was to some extent alleviated when they were used in combination with 2,4-D since rather fewer of the embryos and clumps turned white.

Table 4.4. Effect of sucrose on the maintenance of somatic embryos

Nature of somatic embryos	Medium		% somatic embryos showing white appearance	% primary embryos producing secondary embryos
	sucrose (gl^{-1})	2,4-D (mgl^{-1})		
Individual	20	0	40	10
	40	0	100	0
	60	0	100	0
	20	4.0	20	20
	40	4.0	70	10
	60	4.0	70	0
Clumps	20	0	20	0
	40	0	90	10
	60	0	90	10
	20	4.0	0	90
	40	4.0	30	70
	60	4.0	60	40

Key :

Number of replicates : 10

Basal medium : MS

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\mu Mm^{-2}s^{-1}$PAR

4.2.4. Studies to investigate an appropriate subculture method

An investigation aimed at the maintenance of embryogenic competence of somatic embryos once clumps had been established, involved the use of different subculturing regimes (2,4,6 and 8 weeks) on medium supplemented with 4.0 mg^{-1} 2,4-D. This level of 2,4-D was chosen on the basis of the results described in Section 3.3.1.1) showing that it was the optimum level for proliferating somatic embryos Plates 4.2A,B show the proliferation process of secondary somatic embryos.

The results show that the shorter the subculture intervals, the lower the frequency of somatic embryos turning white and showing loss of embryogenic competence (Table 4.5). Although, two-week and four-week subculture intervals resulted in all of the clumps of somatic embryos being healthy and capable of producing secondary embryos, the two-week subculture interval favoured the growth of nonembryogenic tissues which overgrew the embryogenic tissues.

Even with the optimal four-week subculture regime the growth of nonembryogenic tissues was not entirely eliminated and so it was necessary to discard these tissues, together with mature somatic embryos possessing green cotyledons at each subculture.

It was also noted that continuous maintenance on medium containing 4.0 mgl^{-1} 2,4-D for three or four passages led to the gradual loss of embryogenic competence. Transfer to medium containing a lower concentration of 2,4-D (2.0 mgl^{-1}) after such passages for a single passage was therefore necessary for the long-term maintenance

Table 4.5. Effect of subculture intervals on embryogenic potential

subculture intervals (weeks)	% clumps of somatic embryos showing embryogenic competence
2	100
4	100
6	70
8	40

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod,

$30 \mu Mm^{-2}s^{-1}$PAR

passages for a single passage was therefore necessary for the long-term maintenance of embryogenic competence. Medium with 2,4-D at a concentration lower than 2.0 mgl^{-1} accelerated the growth of somatic embryos so that they regenerated into plantlets.

4.3. DISCUSSION

The results showed that production of somatic embryos of cassava could be maintained on a long-term basis by preventing embryo germination and allowing embryo proliferation. The ability of cassava primary somatic embryos to undergo further embryogenesis (Section 4.3.1) is in agreement with observations showing that plantlets of *Atropa belladonna* (Rashid and Street, 1973), *Brassica napus* (Thomas *et al.*, 1976), carrot (Mc William *et al.*, 1974), *Datura innoxia* (Geier and Kochlenbach, 1973), *Ranunculus sceleratus* (Konar *et al.*, 1972) arising in culture via embryogenesis often undergo further embryogenesis, spontaneously, at the surface. In cassava, however, the productivity of secondary embryogenesis was dependent on the choice of media and subculture regimes which favoured the continuous presence of embryos of particular size and stage of development.

Extensive proliferation of secondary embryos was achieved, therefore, by the visual selection of embryogenic callus at each subculture, by discarding nonembryogenic tissues, roots and shoots and by the use of alternating high and low 2,4-D (4.0 and 2.0 mgl^{-1}) concentrations in the culture medium (Section 4.2.4). Visual observations to select morphogenic tissues prior to transfer proved to be necessary for the maintenance of morphogenic cultures of sugarcane (Chen *et al.*, 1988), *Albizia richardiama* (Tomar and Gupta, 1988), and *Zea diploperennis* (Swedlund, 1983). In cassava, the removal of nonembryogenic tissues is important since they were found to overgrow embryogenic tissues after two or three subcultures.

The beneficial effects of a regular transfer after four passages to a medium containing a lower concentration of 2,4-D (2.0 mgl^{-1}) seem to be in agreement with the

work of Reinert *et al.* (1968, 1970) with carrot showing that the withdrawal of 2,4-D from the medium allowed the reinduction of embryogenesis in carrot cultures which had ceased to form embryos. Chen *et al.*(1988) showed that continued subculture of the compact embryogenic callus on medium containing 3.0 mgl^{-1} 2,4-D caused callus production of sugarcane to decrease and eventually decline to zero by the end of the fourth subculture ; this could be prevented by transfer to a medium containing 1.0 mgl^{-1} 2,4-D after such subcultures. Embryogenic cultures of the other plant species have been maintained by using a combination of 2,4-D and cytokinin since Heyser *et al.*(1983)and Nabors *et al.*(1983) reported a decrease in embryogenic callus and potential for regeneration, when callus was maintained on medium containing 2,4-D alone.

The subculture interval proved to be critical for maintaining cassava embryogenic tissues and a four-week subculture interval seemed to be optimal (Section 4.2.4). A longer interval, led to overgrowth of nonembryogenic tissues and the loss of embryogenic competence. Swedlund and Locy (1988) also showed that the choice of an appropriate subculture interval was critical for the maintenance of embryogenic callus of *Zea diploperennis* cultured on semi solid medium.

The uniformity of somatic embryos during long-term maintenance seemed to be affected by ABA in combination with 2,4-D. The effect of ABA in the maintenance and normalization of somatic embryos has been reported by other investigators. Rajasekaran *et al.*(1987a,b); Quatrano *et al.*(1983) demonstrated that the embryogenic competence of *Pennisetum purpureum* and wheat callus respectively could be partially restored upon subsequent treatment with ABA. When somatic embryos of wheat were cultured in the presence of exogenous ABA, certain proteins such as wheat germ agglutinin, dramatically accumulate within a few days, coupled with germination inhibition

and resumption of the embryo maturation process. In addition, Qureshi *et al.*(1989) demonstrated that the embryogenic callus induced at a lower concentration of ABA (0.25 mg^{-1}) exhibited precocious germination of somatic embryos in contrast to those induced at a higher concentration of ABA (0.5-1,0 mgl^{-1}). They also showed that the effect of ABA varied depending the time of application; more embryogenic callus was produced when the late-stage embryos were cultured on medium in the presence of ABA. In contrast, the addition of ABA to medium for culturing early-stage embryos led to only 20% of them producing embryogenic callus. A different effect of ABA was noted by Kitto and Janick (1985) who showed that the effectiveness of ABA was less at the beginning than at the end of the embryo induction phase. They also reported that ABA retarded embryo development and blocked precocious germination, but did not inhibit embryo initiation. The effect of ABA seems also to be affected by light as Ammirato (1977) reported that in light-grown populations, ABA prevents the greening of cotyledons and permits embryogenic axes to remain unelongated and bear fleshy cotyledons.

The results also showed (Section 4.2.2) that somatic embryos treated with a high concentration of ABA (4.0 mgl^{-1}) became waterlogged and they ceased to undergo further embryogenesis. Their embryogenic competence was not lost, however, since embryogenesis could be reinduced by tranferring them to hormone-free medium. This was to some extent in accord with Kitto and Janick's (1985) observation that the growth-retarding effect of ABA is reversible as demonstrated by the germination and growth of carrot somatic embryos after encapsulation and rehydration.

It was shown that gibberellic acid could not be used for maintaining cassava embryogenic tissues (Section 4.2.2) due to its effect in inducing the growth of the cotyledons of the somatic embryos. Similarly, Ammirato (1977) reported that GA_3 when used at concentrations from 10^{-8} to 10^{-6} M promoted cotyledon development in caraway

somatic embryos, increase the percentage of callused embryos and had little effect on the relative distribution of normal and abnormal embryos. Also, although a particular combination of GA_3 and ABA permitted greening of the cotyledons of carrot somatic embryos, a similar concentration of ABA alone caused the embryos to remain white resulting in an enhancement of normal development; on the other hand GA_3 alone increased the incidence of aberrant embryos (Ammirato, 1977). These results were in agreement with those obtained in Section 4.2.2.

The attempt to maintain cassava embryogenic tissue and somatic embryos by the use of high sucrose concentrations was not successful (Section 4.2.3). Galzy and Compan (1988) demonstrated that different species of the genus *Vitis* required a different level of sucrose (0.3% as opposed to 1.5%) for long-term maintenance. Higher concentrations of sucrose (6% or 12%) have been successfully used to maintain sunflower somatic embryogenic tissues (Chandler and Beard, 1983).

CHAPTER 5

CULTURE OF CASSAVA CELLS AND PROTOPLASTS

5.1. INTRODUCTION

Since *in vitro* plant regeneration is important for the appplication of biotechnological techniques, attempts to regenerate cassava from suspensions, cells and protoplasts were carried out for the following reasons : (a) to improve effiency of embryo production , possibly as a means of propagation, (b) to allow the use of suspensions for *in vitro* selection, (c) to produce large amounts of embryogenic tissue for use with genetic transformation techniques, and (d) to produce a reliable method for regeneration from protoplasts used for fusion and genetic transformation.

5.1.1. Embryogenic suspension cultures

Although a high frequency of embryogenesis (up to 100% depending on the genotype) can be obtained from explants cultured on semi-solid medium, this a laborious system involving the separation and transfer of individual clumps. An alternative system, therefore, is required if somatic embryos are to be induced and regenerated on a large-scale basis. Ammirato (1987), Hussey (1983), Lu *et al.*(1981), Vasil and Vasil (1980) suggested that suspensions provide a system for rapid and large-scale clonal propagation, since in such cultures, proembryogenic clusters and somatic embryos usually separate from each other and float freely in the medium. Large numbers of cells can be moved easily from one vessel to another and through the various treatments cells can be induced to grow into somatic embryos and then into plantlets (Ammirato, 1983). Also, because cells and somatic embryos are bathed by the culture medium and evenly exposed to nutrients and hormones, more precise manipulation of media components, sieving of cells and somatic embryos and control of development is possible (Ammirato, 1983). These properties of embryogenic suspension cultures, and

in particular the presence of free-floating individual somatic embryos, allows the possibility of the mechanical handling and coating of somatic embryos to produce 'artificial seeds'. Fluid drilling (Gray, 1981) has been suggested as one way to deliver large quantities of somatic embryos from the culture flasks to the field (Evans *et al.*, 1981). Artificial seeds have been reported by Kitto and Janick, 1985; the somatic embryos of carrot formed in suspension cultures were coated with polyethylene oxide and dispensed onto teflon sheets after the embryogenic suspensions had been pretreated with ABA (10^{-6} M) during the 14 days of the embryo induction phase.

Selection schemes with embryogenic cultures have given rise to temperature-sensitive carrot variants for studies of differentiation (Breton and Sung, 1982) and stable salt-tolerant *Citrus* embryos and plants (Kochba *et al.*, 1982). Al-Abta *et al.*(1979) pointed out that embryogenic suspension cultures and populations of somatic embryos may be an alternative system for the production of important chemicals, since somatic embryos of celery produced the same flavour compounds as those present in the mature plant but absent from celery callus cultures. Similarly, somatic embryos of cacao produced the same lipids, including cocoa butter, as their zygotic counterparts (Pence *et al.*, 1981).

Cell suspension cultures capable of regenerating plantlets by the process of somatic embryogenesis were first reported in carrot (Steward *et al.*, 1958). There are several factors considered to affect the establishment and maintenance of embryogenic suspension cultures such as the nature of the embryogenic tissue, the concentration of 2,4-D and the subculture regime. The nature of embryogenic tissues as well as the amount of embryogenic tissues formed are influenced by the quality of the explants; shiny and compact embryogenic tissues generally lead to the best result (Bhojwani and Razdan, 1983; Vasil and Vasil, 1980). The amount of 2,4-D is particularly critical during

the period of initiation of suspension cultures of some plant species (Dalton, 1988; Gamborg *et al.*, 1983; Harris *et al.*, 1988; Kumar *et al.*, 1988; Stuart and Strickland, 1984; Vasil, 1980). The removal of supernatant medium containing enlarged and vacuolated non embryogenic cells and other cellular debris is critical during the early stages of the establishment of embryogenic suspensions and during their maintenance (Bayliss, 1977; Sunderland, 1977; Evans and Gamborg, 1982).

5.1.2. Somatic embryogenesis in single-cell cultures

Attempts to culture single cells as opposed to the crude mixture of cells and aggregates formed in suspension cultures aroused interest because of the fact that single cell cultures allow more precise manipulation. The development of each cell can be followed so that selection and cell transformation which is a potential technique for interspecific gene transfer might be carried out. Nitrate Reductase (NR)-deficient cell lines have been isolated by selecting single cells for chlorate resistance (Muller and Grafe, 1978). The success of manipulating carrot single cells which could survive and divide following microinjection (Nomura and Komamine, 1986) should ease the possibility of the induction of mutants and variants.

It has been reported that somatic embryogenesis in cultured cells is also a useful system for research on the development of a whole plant from a single cell (Nomura and Komamine, 1985). Button and Bohta (1975) argued that practically every cell in citrus callus was naturally embryogenic and that, where somatic embryos developed, they were derived from single cells. This callus retained its embryogenic capacity after numerous subcultures. Nomura and Komamine (1985) have established a system in

which single cells of carrot differentiated to embryos at a high frequency. They indicated that there are at least two phases in the differentiation of somatic embryos from single cells; the progression of the first phase required exogenous auxin, whereas that of the second phase was inhibited by the same growth regulator.

In general, the frequency of embryogenesis from single cells is still very low. Most single-cell cultures of plant species, either form callus only or undergo direct organogenesis, and rarely undergo somatic embryogenesis. Nomura and Komamine (1985) pointed out that the cause of the low frequency of embryogensis from carrot single cells was that there were several types of single cells in the fraction (spherical, oval shape and elongated cells) and some of these single cells (about 90% of the oval cells and 100% of the elongated cells) could not form embryogenic cell clusters under the culture conditions used. To improve this frequency, therefore, it was necessary to eliminate the oval and elongated cells from the initial population.

Different methods for isolating single cells from plant material have been reported. Mechanical isolation was considered to have at least two distinct advantages over the enzymatic method (Bhojwani and Razdan, 1983): it eliminates the exposure of cells to the harmful effects of enzymes, and the cells need not be plasmolysed. In the enzymatic method of cell isolation, it is necessary to provide the cells with osmotic protection since the enzyme used to isolate the cells not only degrades the middle lamella but also weakens the cell wall.

The success of single-cell cultures is affected by the composition of the medium and the initial cell plating density. A medium containing a combination of auxin and cytokinin is normally used to induce single cells to differentiate to somatic embryos (Bhojwani and Razdan, 1983; Rubos, 1985). Button and Bohta (1975), however, showed that the

induction and maintenance of embryogenic citrus single-cell cultures did not require the addition of any growth regulator to the basal medium.

The cell density effect on cell division has been explained on the basis that cells synthesize certain compounds, which should reach a threshold value before the cell can embark on division. The cells continue to lose these metabolites into the medium until an equilibrium is reached between the cell and the medium; at a high cell density the equilibrium is attained much earlier than at low cell density, and hence the lag phase is shorter under the former condition. Below a critical cell density, the equilibrium is never reached and the cells fail to divide (Bhojwani and Razdan, 1983; Vasil and Vasil, 1980).

5.1.3. Somatic embryogenesis in protoplast cultures derived from embryogenic suspension cultures

For any crop species, it is important that a reliable method for plant regeneration from protoplasts should be developed if the full range of genetic manipulation procedures, including somatic cell hybridization and transformation by direct gene transfer, is to be exploited for plant improvement. Rapidly growing cell cultures can be one of the most suitable sources of protoplasts, particularly where difficulties have been encountered in isolating culturable protoplasts from leaves or from other parts of the plant. In the present investigation it was considered that, since embryogenically competent cells appear to be highly localized in the immature region of the plant, embryogenic suspension cultures or somatic embryos might provide the most useful alternative sources of protoplasts capable of plant regeneration.

Several investigators have stressed the importance of using embryogenic cultures to obtain somatic embryos regenerated from protoplasts. Vasil and Vasil (1980) have used embryogenic cultures to overcome the recalcitrance of *Pennisetum americanum* and they successfully obtained somatic embryos which eventually regenerated into plantlets. Somatic embryos and plant regeneration have also been obtained from embryogenic cell suspension cultures of *Picea glauca* (Attree, *et al.*, 1987; Bekkaoui *et al.*, 1987), of *Pseudotsuga menziesii* (Gupta *et al.*, 1986), of *Pinus taeda* (Gupta and Durzan, 1987), of *Panicum maximum* (Lu *et al.*, 1981), of *Pennisetum purpureum* (Vasil *et al.*, 1983) and of *Larix x eurolepis* (Klimaszewska, 1989); whereas only microcalli could be obtained with embryogenic callus-derived protoplasts of *Zoysia japonica* (Asano, 1989) and of *Abies alba* (Lang and Kohlenbach, 1989).

Regeneration of protoplasts isolated from both embryogenic callus and embryogenic suspension cultures seems to be affected by several factors including the plating density, the composition of medium and culture conditions. Various methods have been used to facilitate the penetration of enzyme solution, which is essential for effective digestion to obtain a high protoplast yield. Preplasmolysis in a salt solution containing 0.44 M- 0.50 M mannitol prior to enzyme incubation stabilized and improved the quality of released protoplasts from embryogenic suspension cultures of *Picea glauca* (Attree *et al.*, *loc.cit*) and of *Laryx x eurolepis* (Klimaszewska, *loc.cit.*). In Gramineae, however, high protoplast yields could be obtained without preplasmolysis, simply by incubating the embryogenic cells in enzyme solution containing Onozuka Cellulase R-10 alone which pH had been adjusted to 5.6-5.8 (Vasil and Vasil, 1984), since the activity of the enzymes is considered to be pH dependent (Bhojwani and Razdan, 1983).

As in single-cell cultures, the initial plating density of embryogenic culture-derived protoplasts has a profound effect on the plating efficiency. Depending on the genotype,

these protoplasts are generally cultured at a density of 1 x 10^4 to 1 x 10^5 protoplasts ml^{-1}. A higher plating density (1-4x10^5), however, was required by protoplasts of the Gramineae (Vasil and Vasil, 1984) to undergo division.

As an osmotic stabilizer, mannitol 0.44-0.60 M seems to be used more widely in preparations of protoplasts from embryogenic suspension cultures. In culture medium, the type of osmoticum used varies depending on genotypes; mannitol (0.4-0.5 M) has been used for culturing protoplasts of *Laryx x eurolepis* (Klimaszewska, 1989) and of *Abies alba* (Lang and Kohlenbach, 1989), while glucose has been used for culturing those of *Picea glauca* (Atrree *et al.*, 1987; Bekkaoui *et al.*, 1987), and of the Gramineae (Vasil and Vasil, 1984). Although the composition of hormones varied between culture of plant species, 2,4-D alone or in combination with BAP or zeatin was most often used in protoplast cultures isolated from embryogenic cultures. In *Picea glauca* (Attree *et al.*, *loc.cit.*) and some species of the Gramineae (Vasil and Vasil, *loc.cit.*), the presence of 2,4-D alone (0.5-2.5 mgl^{-1} and 0.25-1.0 mgl^{-1} respectively) was sufficient to induce cell division.

Embryogenic culture-derived protoplasts generally require incubation in the dark at 25°C to undergo cell division and form microcalli which eventually regenerate to plantlets. A higher temperature (27 and 28°C), however, was reported to favour the development of protoplasts of the Gramineae (Vasil and Vasil, *loc.cit.*) and of *Zoysia japonica* (Asano, 1989) respectively.

5.2. MATERIALS AND METHODS

5.2.1. Initiation and maintenance of suspension cultures

Embryogenic tissues induced from leaf lobes of the cultivar CMC 76 on semi-solid medium supplemented with 4.0 mgl^{-1} 2,4-D were placed in 250 ml Erlenmeyer flasks containing 40 ml MS liquid medium. The cultures were then placed on an orbital shaker in the light (see Chapter 2). The resulting suspension cultures were maintained by pouring off the old medium, after the cells had been allowed to settle for five minutes. 10 ml of the cell suspension was inoculated to 20 ml new fresh medium containing 2.0 mgl^{-1} 2,4-D. This procedure was repeated every two weeks.

5.2.2. Isolation and culture of single cells

The leaves were dipped in 70% (v/v) ethanol for 30 seconds before they were transferred to sodium hypochlorite solution (1.5% available chlorine) for three minutes. The further sterilisation procedure was as described in Chapter 2. Surface sterilized leaves were then brushed to ease the release of mesophyll cells using a sterile brush after the removal of the midrib and then cut into small pieces (2.0 x 2.0 mm) They were then macerated either mechanically or enzymatically. In the latter case, 3 g of the leaf pieces were incubated in 30 ml enzyme solution in 250 ml Erlenmeyer flasks placed on an orbital shaker for 18 hours then vacuum infused. They were then filtered

Table 5.1. Composition of modified CL (Cell layer) medium

Macronutrients	mgl^{-1}
KNO_3	7600
$CaCl_2.2H_2O$	1760
$MgSO_4.7H_2O$	1480
KH_2PO_4	680
Micronutrients	
KI	0.42
H_3BO_3	3.10
$MnCl_2.4H_2O$	9.90
$ZnSO_4.7H_2O$	4.60
$Na^2MoO_4.2H_2O$	0.13
$CuSO_4.5H_2O$	0.013
$CoSO_4.7H_2O$	0.015
Na_2.EDTA	18.50
$FeSO_4.7H_2O$	13.90
Organic adenda	
Pyridoxine.HCl	0.5
Thiamine.HCl	0.5
Folic acid	0.5
Glycine	2.0
Nicotinic acid	5.0
Casein hydrolysate	50.0
Osmoticum	**M**
Sucrose	0.100
myo-inositol	0.013
D-manitol	0.013
Sorbitol	0.013
Xylitol	0.013
Other	
Agarose	0.2%
pH	5.7

After Shahin and Shepard (1980)

through a 1.5 x 1.5 mm mesh prior to centrifugation at 150 g for 5 minutes. The pellet was washed with washing medium (half-strength MS medium supplemented with 2% sucrose) by recentrifugation at the same speed. The pellet was then plated by spreading on semi- solid medium or incubated, after dispersal in MS liquid medium in 5 cm Petri dishes, in the dark for the first two weeks then in the light (16 hours photoperiod, 40 $\mu Mm^{-2}s^{-1}$ PAR).

5.2.3. Isolation and culture of protoplasts

Somatic embryos (2-3 g) were suspended in 20 ml of pretreatment solution (see Table 5.2) in a Petri dish and incubated for one hour at room temperature. To remove this solution, they were then passed through a 1.5 x 1.5 mm mesh before they were resuspended in 20 ml of enzyme solution in a Petri dish. After sealing the Petri dish with Parafilm and covering with aluminium foil, the mixture was agitated on a shaker at 80 rpm. During the incubation, the yield of protoplasts was checked at two-hour intervals to obtain the optimum value. After 18 hours, at which the optimum protoplast yield was obtained, the mixture was passed through a 1.5 x 1.5 mm mesh to remove undigested tissue fragments. By using a Pasteur pipette, the filtered suspension collected in a Petri dish was passed through the mesh several times to release the trapped protoplasts. The suspension was then passed through a smaller mesh (0.5 x 0.5 mm) to remove larger debris before it was centrifuged at 150 g for 5 minutes. The pellet was suspended in a washing medium (see Table 5.2) and recentrifuged at the same speed for another 5 minutes. The final protoplast suspension was gently resupended on 14.5% sucrose solution in sterile double-distilled water and recentrifuged at 100 g for 5 minutes. The protoplasts which formed a band on top of the solution were collected and either suspended in 5 ml liquid medium or dispersed on 5 ml semi solid medium at different densities in Falcon dishes. The dishes containing protoplasts were then sealed and

Table 5.2. Composition of media used for preparation of protoplast cultures

Pretreatment solution in water	: Mannitol	0.5 M
	$CaCl_2.2H_2O$	5.0 mM
	MES	5.0 mM
Enzyme solution in water	: Cellulase (w/v)	1.5%
	Macerozyme (w/v) 0.5%	
	Mannitol	0.5 M
	$CaCl_2.2H_2O$	5.0 mM
	MES	5.0 mM
Basal medium	: MS	
	Mannitol	0.5 M
	$CaCl_2.2H_2O$	5.0 mM
	MES	5.0 mM
Other	: Agarose	0.5%
	pH	5.7

placed in a 'Stewart' plastic (England) container (nine dishes in one container) to which sterile water was added to provide a saturated atmosphere. After covering the container with aluminium foil, the cultures were incubated at $25\pm1^{o}C$.

5.3. RESULTS

5.3.1. Induction and proliferation of somatic embryos in suspension cultures

5.3.1.1. The effect of 2,4-D and zeatin on the induction of somatic embryos from suspension cultures

A comparison was made between the ability of friable callus and embryogenic tissues derived from leaf lobes to initiate embryogenic suspension cultures. Two different amounts of initial fresh weight (1.5 and 3.5 g), six different media supplemented with 2,4-D or zeatin alone or in combination and two different speeds of rotation (90 and 120 rpm) were tested to investigate their effect on the induction of embryogenesis in the suspension cultures. The volume of medium used was 40 ml (see details in Section 5.2.1).

After the suspension cultures were established (eight weeks), two different subculture procedures were employed : some batches were subcultured with medium containing the same concentration of hormones as used for the suspension initiation, while other batches were subcultured with a lower concentration of hormones. Packed-cell volumes were measured to determine growth in each treatment and the production of somatic embryos were scored after they were plated out on MS semi-solid medium containing 2.0 mgl^{-1} 2,4-D.

Four days after initiation, observation of cultures treated with different hormones as previously mentioned indicated that very few cells were released into the medium irrespective of treatment tested. Observation of two-week old cultures revealed that the highest number of living cells and of spherical embryogenic cells was obtained with the

medium containing 4.0 mgl-*1* 2,4-D alone (Plates 5.4A shows the division of embryogenic cells stained with FDA). The subculturing of these cultures with medium containing the same concentration of 2,4-D, however, led to the reduction in the number of living cells. Moreover, the cultures turned brown earlier than those with a lower concentration of 2,4-D. On the other hand, lowering 2,4-D concentration to 2.0 mgl^{-1} at every subculture after the initial establishment of the suspension cultures led to an increase of the number of living cells (Table 5.3) and the cell fresh weight (Table 5.4). Medium with a combination of 4.0 mgl^{-1} 2,4-D and 0.02 mgl^{-1} zeatin resulted in the second highest yield in terms of cell fresh weight. However, the stock suspension cultures on this medium started forming large aggregates of friable callus by three months (four subcultures) which resulting in the suspension becoming very thin.

Medium containing an initial concentration of 2,4-D at 0.4 mgl^{-1} either alone or in combination with 0.02 mgl^{-1} zeatin resulted in a suspension composed of more elongated cells. Zeatin, in this case in a combination with a low 2,4-D concentration, therefore, did not seem to induce embryogenesis. On the other hand, medium containing a high concentration of 2,4-D (8.0 mgl^{-1}) produced a brown suspension consisting of a high proportion of dead cells.

It seemed that the higher speed of rotation was required for the induction of embryogenic suspension cultures. Cultures rotated at 90 rpm only produced friable callus, comprised of elongated cells and they failed to produce embryogenic cells. By contrast, those rotated at 120 rpm were composed of clusters of isodiametric cells and they produced somatic embryos either floating freely or in clumps.

The type of suspension cultures initiated also depended on the type of inoculum that was used. Friable callus used for the initiation of the suspension cultures gave rise to

Table 5.3. The effect of 2,4-D and zeatin on the number of living cells in suspension cultures

Medium (mgl^{-1})	Number of living cells ($x10^2$ per ml)	
	2 weeks	4 weeks
2.0 2,4-D	4.2	9.8
4.0 2,4-D	10.4	11.7
4.0 2,4-D + 0.02 zeatin	6.9	9.2
8.0 2,4-D	6.2	8.9

Key

Number of counts : 6

Basal medium : MS supplemented with 2% sucrose

Initial inoculum : 1.5 g embryogenic tissues

Speed of rotation : 120 rpm

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\,\mu Mm^{-2}s^{-1}$PAR

Table 5.4. The effect of 2,4-D and zeatin on cell fresh weight in suspension cultures

Initial concentration (mgl^{-1})	Fresh weight ($mgml^{-1}$)			
	9 wks	11 wks	13 wks	15 wks
2.0 2,4-D	24.6^{a}	26.5^{a}	28.9^{a}	22.6^{a}
4.0 2,4-D	80.9^{b}	81.1^{b}	77.9^{b}	75.4^{b}
4.0 2,4-D + 0.02 zeatin	39.4^{c}	34.8^{c}	33.8^{c}	25.5^{c}
8.0 2,4-D	6.6^{d}	8.5^{d}	9.1^{d}	9.7^{d}

Values with same superscript do not differ significantly ($P<0.05$)

Key

Number of replicates : 3

Basal medium : MS supplemented with 2% sucrose

Initial inoculum : 1.5 g embryogenic tissues

Speed of rotation : 120 rpm

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\mu Mm^{-2}s^{-1}$ PAR

aggregates of friable non-embryogenic callus and comprised largely of elongated cells. In contrast, embryogenic callus, irrespective of the initial amount of tissue used, resulted in the formation a suspension composed of clusters of isodiametric embryogenic cells and somatic embryos. Smaller inocula of embryogenic callus (1.5 g) seemed to be better than those of 3.5 g in terms of continuation or maintenance of the suspension cultures. The latter produced cultures which required subculturing three times within the first two weeks or otherwise the cultures turned brown and lost their embryogenic potential.

The formation of somatic embryos could be observed when the cultures were five weeks old. Besides the formation of young somatic embryos possessing cotyledons, small compact spherical aggregates were also observed. An initial medium supplemented with 4.0 mgl^{-1} 2,4-D resulted in the highest frequency of embryogenesis, followed by the medium with 2.0 mgl^{-1} 2,4-D, while that with 8.0 mgl^{-1} 2,4-D gave rise to a large amount of smooth spherical aggregates which eventually regenerated into roots following transfer to hormone-free semi-solid medium. Plate 5.3D shows different types of tissues obtained from suspension cultures following transfer to hormone-free medium.

5.3.1.2. The effect of the type of flasks on the induction of embryogenesis of cultured cells

Having found the optimal concentration of 2,4-D for the induction of embryogenic suspension cultures and the optimal inoculum size, shaking rate, and subculture procedure (Section 5.3.1.1), the effect of modified flasks (Plate 5.1) was investigated. The flasks were modified to increase the agitation and sheering forces within the cultures. The procedures and observations carried out were the same as those in the previous section.

The use of the modified flasks resulted in the earlier establishment of suspension cultures than with those the ordinary flasks. Samples taken from the suspension cultures in the modified flasks, however, revealed that the density of the cultures was not caused by the actively dividing cells but mostly caused by fragmentation of the tissues which resulted in a large amount of debris in the medium. The mean number of living cells observed in one view of microscope showed that only 20-30 cell were alive, while those cultured in ordinary flasks showed 80-100 cells most of which formed embryogenic clusters of appproximately 30 cells in each cluster.

5.3.1.3. The effect of 2,4-D, NAA, and BAP on the frequency of embryogenesis in embryogenic tissues derived from suspension cultures

Suspensions containing individual somatic embryos or embryogenic clumps obtained from the experiments described in Sections 5.3.1.1 and 5.3.1.2 were fractionated into three grades by passing them through two different size of sieve (1.5 x 1.5 mm and 0.5 x 0.5 mm mesh. The different fractions of embryos or embryogenic clumps are shown in Plates 5.1B-D.. They were then plated out on medium solidified with 0.5% (w/v) agarose supplemented with either 2,4-D, NAA, or BAP alone or in combination as summarized in Table 5.5. The frequency of secondary embryogenesis and the numbers of secondary embryos produced per clump were scored.

Of the media tested, a combination of 2.0 mgl^{-1} 2,4-D and 2.0 mgl^{-1} BAP gave rise to the highest number of secondary embryos produced per clump irrespective of the size

Table 5.5. Summary of media used for plating fractionated embryogenic clumps

	Hormones (mgl^{-1})		
	BAP	NAA	2,4-D
1	0.1	-	-
2	0.1	-	0.01
3	-	2.0	-
4	1.0	2.0	-
5	2.0	2.0	-
6	-	-	2.0
7	1.0	-	2.0
8	2.0	-	2.0

Key

Basal medium : MS supplemented with 2% sucrose

Solidifying agent : agarose

Table 5.6. Effect of NAA, 2,4-D and BAP on regeneration and production of secondary embryos from fractionated embryos obtained from suspension cultures

Fraction of somatic embryos	hormone composition of medium (mgl^{-1})			% clumps of embryos producing secondary embryos	number of secondary embryos per clump	total secondary embryo production
	BAP	NAA	2,4-D			
I	0.1	-	-	20	1	2
	0.1		0.01	60	2	12
	-	2.0	-	20	1	2
	1.0	2.0	-	20	2	4
	2.0	2.0	-	20	2	4
	-	-	2.0	40	1	4
	1.0	-	2.0	40	6	24
	2.0	-	2.0	40	12	48
II	0.1	-	-	10	3	3
	0.1	-	0.01	50	3	15
	-	2.0	-	10	6	6
	1.0	2.0	-	30	2	6
	2.0	2.0	-	20	2	4
	-	-	2.0	20	3	6
	1.0	-	2.0	10	4	4
	2.0	-	2.0	10	6	6
III	0.1	-	-	30	1	3
	0.1	-	0.01	30	2	6
	-	2.0	-	40	1	4
	1.0	2.0	-	30	2	6
	2.0	2.0	-	20	3	6
	-	-	2.0	20	1	2
	1.0	-	2.0	30	2	6
	2.0	-	2.0	30	5	15

Key

Number of replicates : 10

Basal medium : MS supplemented with 2% sucrose

Size of fraction : < 0.5 mm (Fraction III)

0.5-1.5 mm (Fraction II)

> 1.5 mm (Fraction I)

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod,

$30\ \mu Mm^{-2}s^{-1}$ PAR

Plate 5.1.

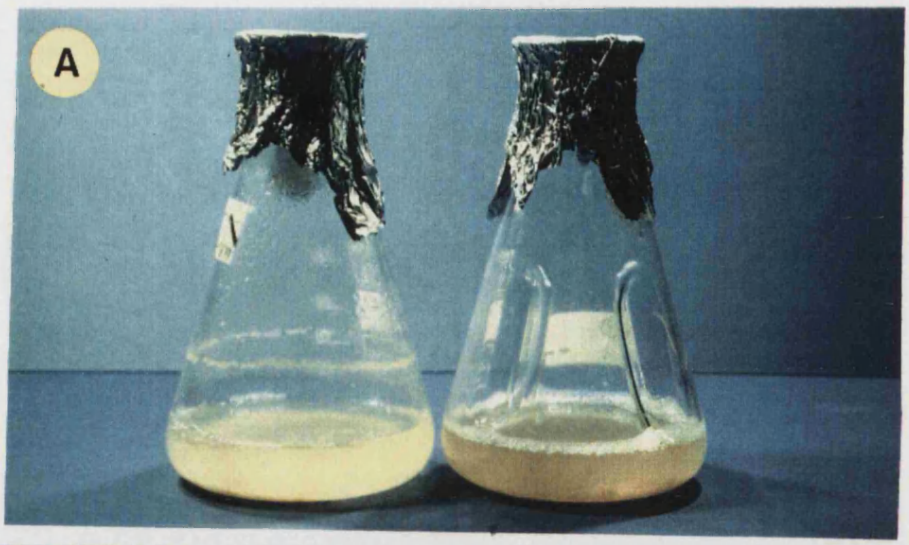
The induction and formation of somatic embryos from embryogenic suspension cultures

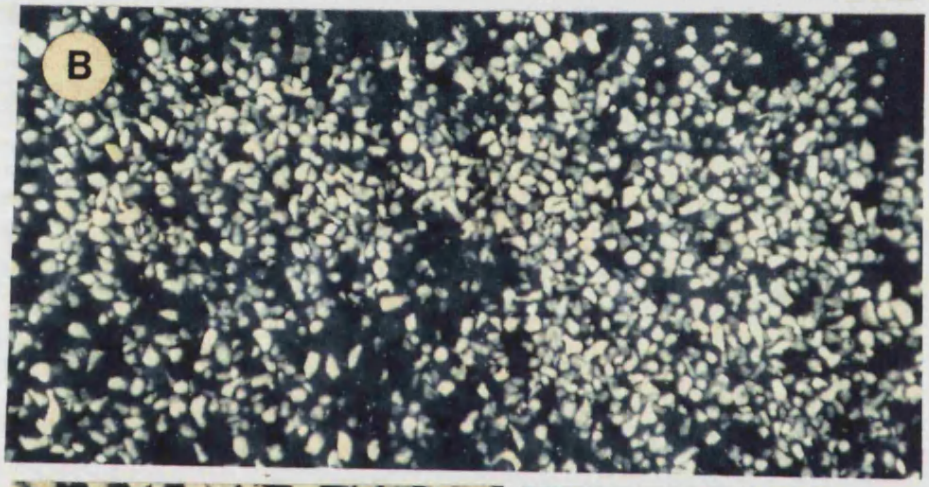
A. Suspension cultures in two different types flasks. x4.5.

B. The smallest size of embryogenic populations (Fraction III) fractionated from suspension cultures (<0.5 mm). x6.

C. The medium size of embryogenic populations (Fraction II) fractionated from suspension cultures (0.5-1.5 mm). x6.

D. The largest size of embryogenic populations (Fraction I) fractionated from suspension cultures (>1.5 mm). x6.


A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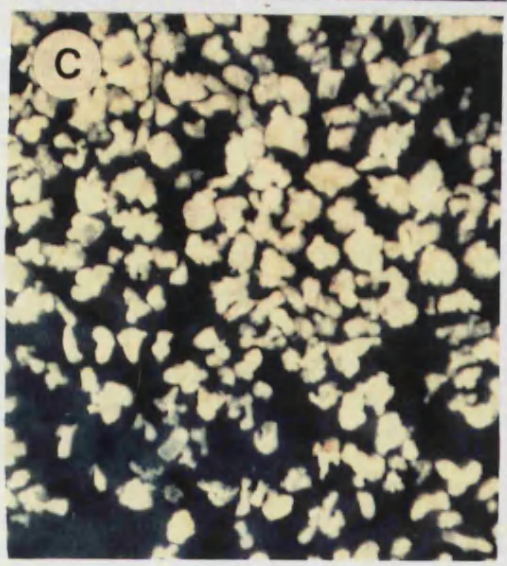
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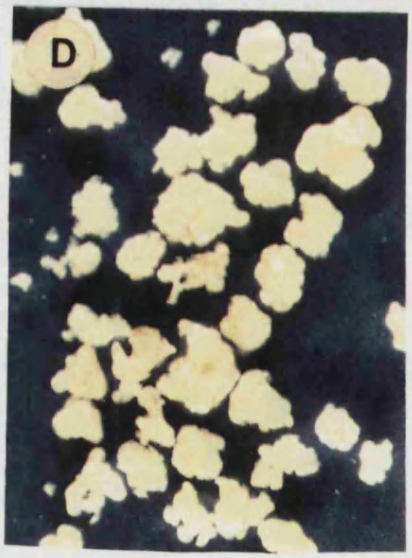

D

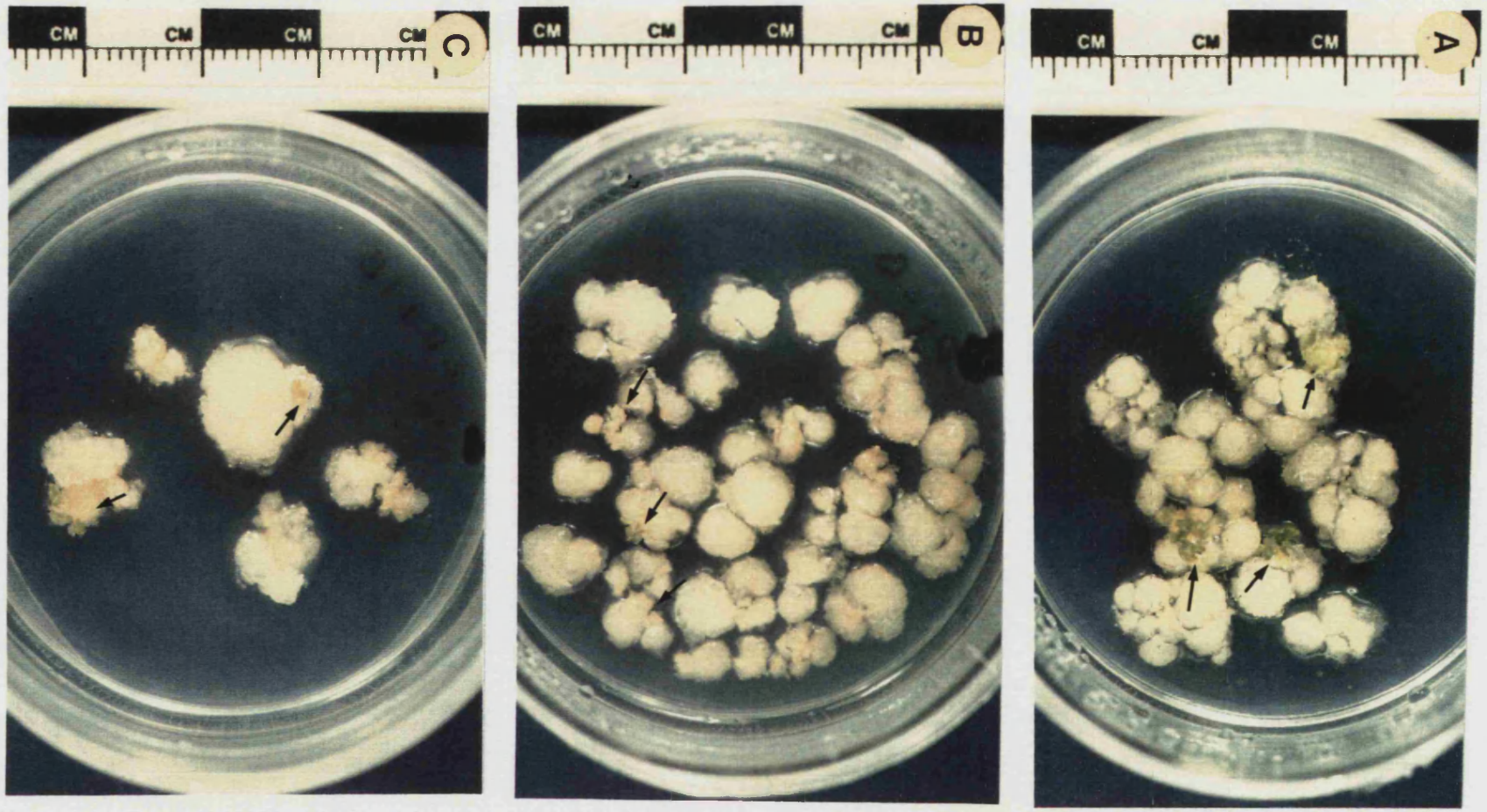
Plate 5.2.

Regeneration of fractionated embryogenic clumps from suspension cultures on medium supplemented with 2.0 mgl^{-1} 2,4-D and 1.0 mgl^{-1} BAP

A. Regeneration of embryogenic clumps from fraction III. x4.5.

B. Regeneration of embryogenic clumps from fraction II. x4.5.

C. Regeneration of embryogenic clumps from fraction I. x4.5.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A

Plate 5.3.

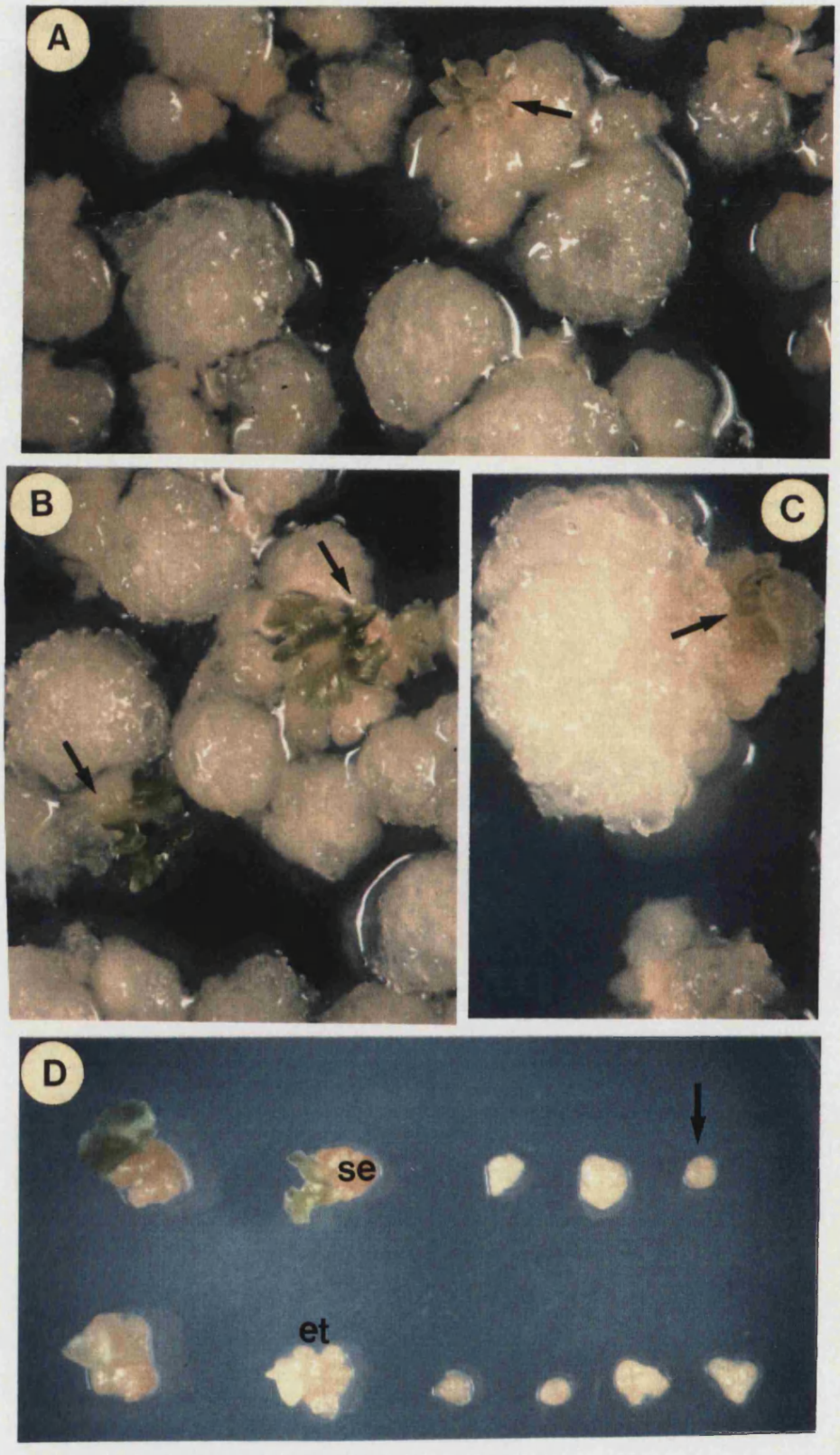
Regeneration of embryogenic clumps from suspension cultures on semi-solid medium

A. Regeneration of embryogenic clumps from fraction III on medium containing 2.0 mgl^{-1} 2,4-D and 1.0 mgl^{-1} BAP. x6

B. Regeneration of embryogenic clumps from fraction II on medium containing 2.0 mgl^{-1} 2,4-D and 1.0 mgl^{-1} BAP. x6.

C. Regeneration of embryogenic clumps from fraction I on medium containing 2.0 mgl^{-1} 2,4-D and 1.0 mgl^{-1} BAP. x6

D. Types of tissues formed in suspension cultures containing 4.0 mgl^{-1} 2,4-D. x6.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of clumps, but the largest size of clumps seemed to be more productive. Plates 5.2 and 5.3 show the regeneration of fractionated embryogenic clumps following transfer to semi-solid medium.

Although medium supplemented with a combination of 0.01 mgl^{-1} 2,4-D and 0.1 mgl^{-1} BAP resulted in the highest frequency of clumps producing secondary embryos, the overall productivity was very low resulting in a low total somatic embryo production.

It seemed that combining either NAA or 2,4-D with BAP led to increases in the total production of somatic embryos (Table 5.6), particularly if BAP was combined with 2,4-D for culturing the somatic embryos of fraction I. It seemed, therefore, that 2,4-D was slightly superior to NAA with regard to the induction of secondary embryogenesis.

5.3.2. Culture of single cells isolated from cassava leaves

Leaves at two different stage of development (unexpanded and well expanded leaves) were used as the sources of single cells. The leaves were macerated either mechanically (using either a glass homogenizer or mortar and pestle) or enzymically or both methods were used, one following the other.

5.3.2.1. Culture of mechanically isolated single cells

To investigate the effect of different methods for isolating single cells mechanically on the yield of cells, the leaves were macerated either with a glass homogenizer or a mortar and pestle. Detailed procedures are described in Section 5.2.

Single cells could only be obtained from well-expanded leaves although the yield was very low. The yield could be slightly increased by macerating leaves with a mortar and pestle.

No cell division was observed in any cultures after incubation in the dark for 30 days.

5.3.2.2. Culture of enzymically isolated single cells

Due to the fact that the yield of single cells obtained by the use of mechanical methods was low, the leaves were macerated enzymically. Three different concentrations of Macerozyme (0.5%, 1.0% and 1.5%) to which was added a solution comprising of 0.5% PVP either with 10 mM EDTA (Otsuki and Takebe, 1965) or without were tested. After 18 hours incubation, they were either directly centrifuged or ground with a glass homogenizer prior to centrifugation.

The highest yield of single cells was obtained from leaves macerated in 1.5% Macerozyme and EDTA solution. Adding EDTA irrespective of the concentration of enzyme used resulted in a higher single cell yield.

Grinding the leaves with a glass homogenizer after enzyme maceration increased the yield of single cells to 6.2×10^2 cells per ml. Plate 5.4B shows freshly isolated single cells from leaves cassava plants grown in the greenhouse.

5.3.3. Culture of protoplasts isolated from somatic embryos of cassava

In view of the difficulties in obtaining single cells from leaves, somatic embryos were used as a source of protoplasts. The somatic embryos were incubated in a pretreatment solution prior to digestion in enzyme solution. The procedure of protoplast isolation is described in Section 5.2.

5.3.3.1. The effect of the composition of medium on the development of protoplasts

Five different media containing the same composition of hormones (0.2 mgl^{-1} 2,4-D + 0.6 mgl^{-1} NAA + 0.8 mgl^{-1} BAP) but differing in the amount of myo-inositol (100 mgl^{-1} or 200 mgl^{-1}), the concentration of macroelements (standard or plus half-strength MS macroelements), or in the type of osmotic agent (glucose or mannitol), and medium containing 1.0 mgl^{-1} 2,4-D and 0.5 mgl^{-1} BAP were tested for culturing protoplasts at a density of 5.2×10^5 protoplasts ml^{-1}. The protoplasts were suspended either in semi-solid medium or in liquid medium either in small drops or covering the surface of the 5 cm Petri dishes.

Of the six different media tested for culturing protoplasts at the density of 5.2×10^5 protoplasts ml^{-1}, only medium supplemented with a combination of 1.0 mgl^{-1} 2,4-D and 0.5 mgl^{-1} BAP gave rise to the change of shape and size of protoplasts. In this medium, particularly when use as small drops of liquid medium, some protoplasts formed buds but no division could be observed although they were still alive by day 40.

Plate 5.4.

Cultures of embryogenic suspension, single cells, and protoplasts on medium supplemented with 2,4-D

A. Division of cells in embryogenic suspension cultures initiated from embryogenic tissues grown on semi-solid medium. The cells were stained with FDA in acetone. x371.

B. Freshly isolated single cells from leaves of cassava cultivar CMC 76. x281.

C. Freshly isolated protoplasts from somatic embryos had been maintained on semi-solid medium. x371.

D. Protoplasts at day four, isolated from somatic embryos. Note debris caused by dead protoplasts. x742.

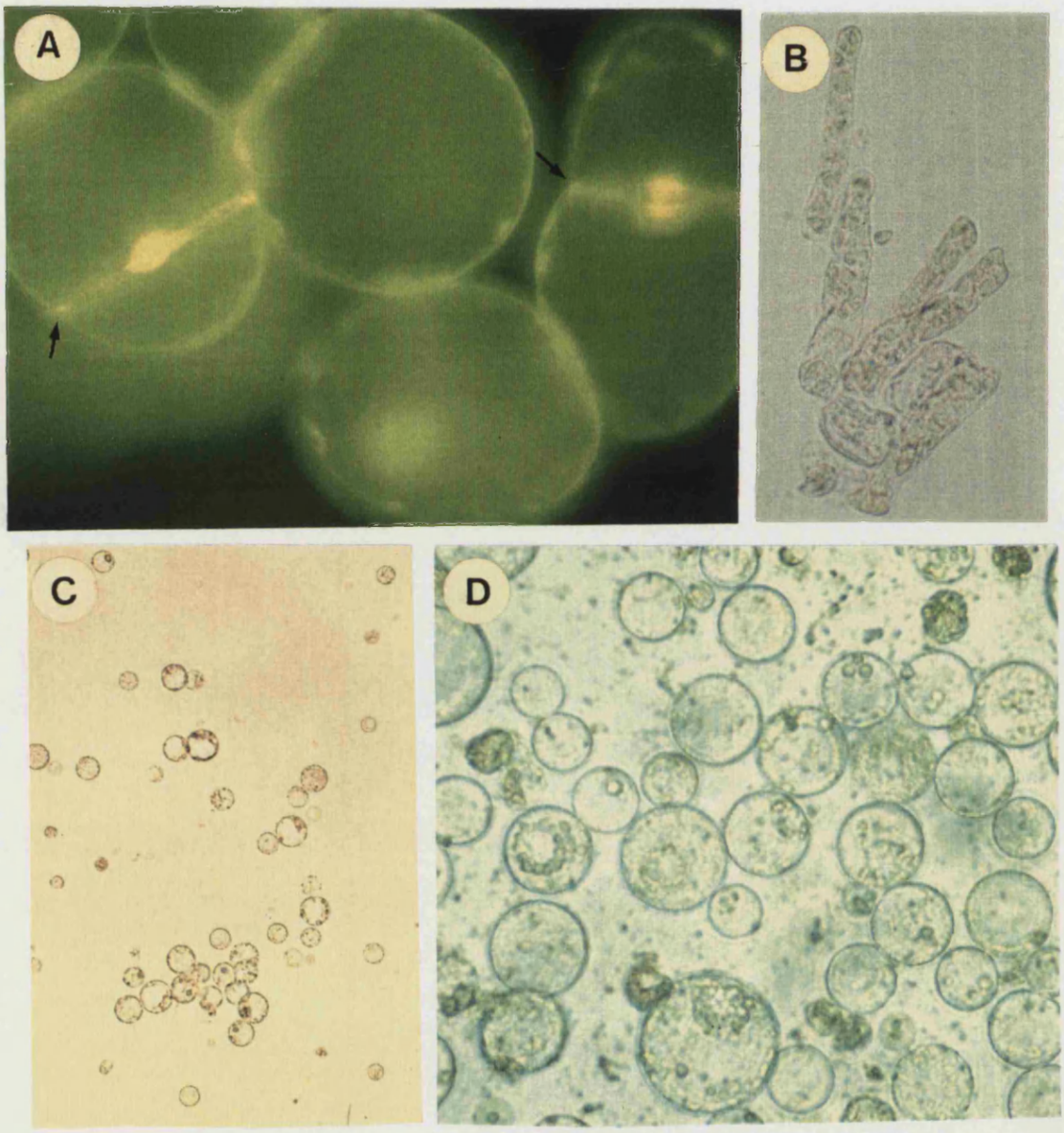
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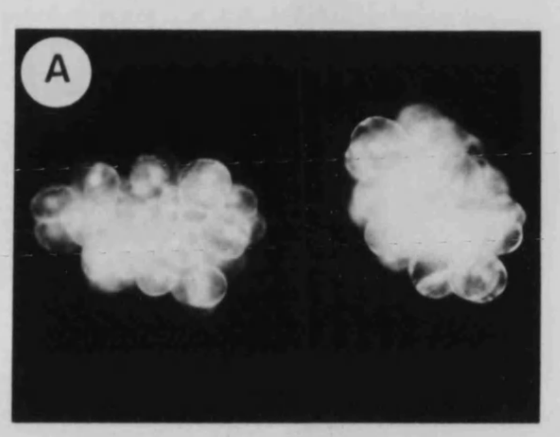
Plate 5.5.

The formation of clusters of isodiametric embryogenic cells in cells in suspension cultures

A. Early stage of the formation of clusters of isodiametric embryogenic cells. x144.

B. Early stage of the formation of clusters of isodiametric embryogenic cells stained with FDA. x144

C. Later stage of the formation of clusters of isodiametric embryogenic cells. x186.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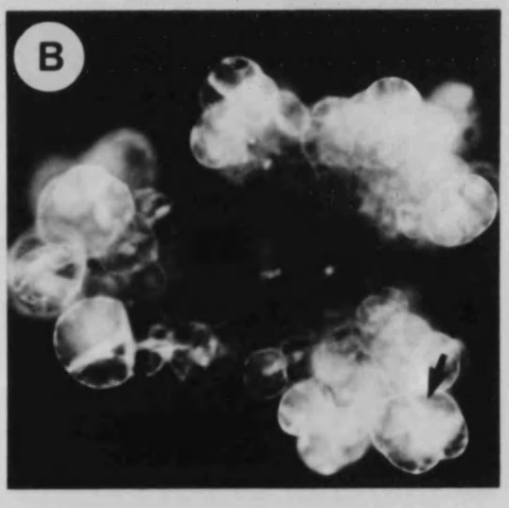
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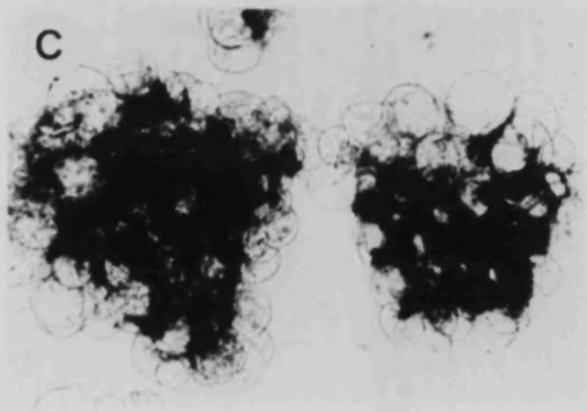

C

Plate 5.6.

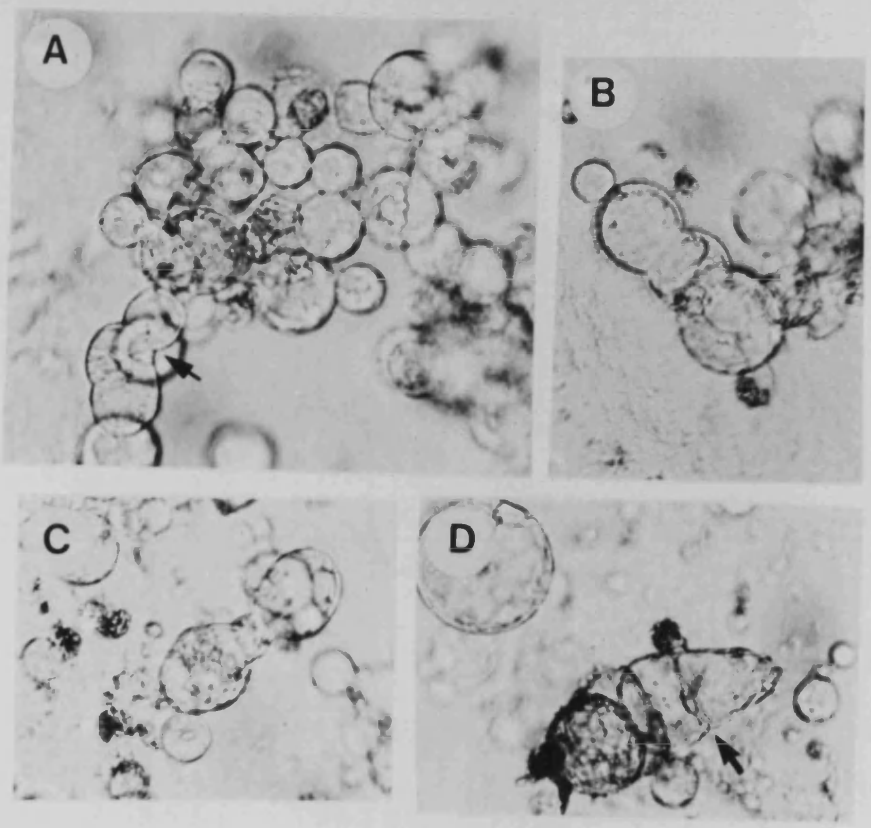
Development of protoplasts cultured in liquid medium containing 2.0 mgl^{-1} 2,4-D, 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} BAP in hanging drops.

A. Budding of protoplasts cultured for 10 days. x330

B. Budding of protoplasts cultured for 10 days. x330

C. Budding of protoplasts cultured for 10 days. x330

D. Possible first division of protoplasts (arrow). x330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5.3.3.2. The effect of plating density on protoplast development

Protoplasts at different densities (2.1 x 10^5, 2.8 x 10^5, 3.8 x 10^5, or 7.0 x 10^5 protoplasts ml^{-1}) were cultured in hanging drops in medium supplemented with a combination of 2.0 mgl^{-1} 2,4-D, 0.5 mgl^{-1} NAA, and 0.5 mgl^{-1} BAP to investigate the optimum density. Plate 5.4C show freshly isolated protoplasts and those cultured four days after isolation.

Observation at day 5 indicated that protoplasts cultured at densities of 2.1 x 10^5, 2.8 x 10^5, and 3.8 x 10^5 protoplasts per ml had formed cell wall which was shown by the change of the shape of protoplasts. During the initial period of culture, whilst the cell wall was reformed, it was also noted that many protoplasts died. The remainder changed shape and underwent budding (Plate 5.6).

A density of 2.1-2.8 x 10^5 protoplasts ml^{-1} proved to be superior to the other densities tested; cell division, although at a very low frequency, could be observed, and the majority of protoplast at this density were budding at day 7 of culture Plate 5.6). The highest density (7.0 x 10^5 protoplasts ml^{-1}) resulted in the highest frequency of dead cells.

5.4. DISCUSSION

As with solid medium, a medium supplemented with 2,4-D alone proved to be the best for the induction of embryogenesis from cultured cell suspensions (Section 5.3.1.1). This result is in agreement with that of Finer and Nagasawa (1988) who successfully used a medium containing 5.0 mgl^{-1} 2,4-D to initiate and maintain embryogenic suspension cultures of soy bean. Gillisen (1983); Sargent and King (1984) on the contrary, showed that the induction of embryogenesis in soy bean required a combination of either 2,4-D or NAA with cytokinin. According to Bergervoet *et al.*(1989) in work with cucumber, NAA was inferior to 2,4-D because it resulted in slower growth and changed the pattern of morphogenesis into organogenesis rather than embryogenesis. In addition, the size of aggregates in the suspension cultures increased as compared to suspensions growing in the presence of 2,4-D. Therefore, 2,4-D was used to disperse the larger aggregates by culturing for one week in medium containing 2,4-D after every three passages in medium containing NAA (Bergervoet *et al.*, 1989). Results (Section 5.3.1) also showed that the addition of zeatin induced the formation of friable callus instead of the formation of embryogenic callus. In contrast, zeatin has been shown to induce embryogenesis at a high frequency in carrot cell suspension cultures (Fujimura and Komamine, 1979).

The concentration of 2,4-D would seem to affect the fresh weight of the cassava cell suspensions, the proliferation of somatic embryos and embryogenic capacity. The suspension cultures seemed to lose their embryogenic potential if concentrations of 4.0

or 8.0 mgl^{-1} 2,4-D were used in the initial medium and also in the media used at every subculture from 30 days after the initiation (Section 5.3.1.1). Fridborg and Eriksson, 1975) also observed that the originally totipotent cultures of carrot completely stopped embryogenesis after eight weeks in medium containing 2,4-D. Loss of embryogenic potential was also observed in *Populus ciliata* suspension cultures after six subcultures in medium containing 0.5 mgl^{-1} 2,4-D (Cheema, 1989).

The 2,4-D concentration also seemed to affect the pattern of morphogenesis as a high 2,4-D concentration (8.0 mgl^{-1}) seemed to cause the formation of more tissues bearing roots only (Section 5.3.1). Halperin (1966) in work with carrot cell suspensions pointed out that, depending upon the particular chemical environment in which the cells are grown, the same cells in culture are capable of giving rise to either a root-bearing clump or to an embryo. He suggested that the formation of proembryos depends upon a rapid rate of cell division under conditions which prevent cell enlargement.

Shaking rate proved to be critical for the initiation of cassava embryogenic cell suspensions (Section 5.3.1.1). Like suspension cultures of other plant species such as *Digitalis obscura* L. (Arrilaga *et al.*, 1987) and cucumber (Bergervoet *et al.*, 1989), embryogenesis of cassava was also induced when cultures were rotated at 120 rpm. Rajasekaran *et al.*(1971) pointed out that growth rate and cell separation are influenced by shaking speed and that a shaking speed satisfactory for one culture strain may be unfavourable for a different strain or species. They also stated that the shaking serves to promote gaseous exchange. According to Tisserat and Mason (1980), mechanical effects in the rotating liquid medium hasten the fragmentation of clumps which occurs when the older cells at the centre of clumps become separated from each other. The result is that the smaller clumps released from a parent clumps are clearly polarized by

virtue of the manner in which they form; each daughter clump bears large vacuolate cells at one end and smaller meristematic cells at the other end.

In addition to hormones and shaking rate, the type of tissues used and the initial inoculum density were also shown to be important for the induction of embryogenesis of cultured cassava cells. Only active embryogenic tissues weighing 1.5-3.5 g inoculated into 40 ml liquid medium gave rise to embryogenesis (Section 5.3.1.1). These results support those of Chee and Cantliffe (1988); Finer and Nagasawa (1988) who showed in work with sweet potato and soy bean respectively that only highly embryogenic tissues at an early ontogenic stage could be used to initiate embryogenic suspension cultures. Chee and Cantliffe (1988) also believe that the non-embryogenic callus cultures never became embryogenic. On the contrary, Cheema (1989) succeeded in employing friable calli to induce cell suspensions in which the growth and development of somatic embryos was retained, but this seems to be rather unusual. With regard to initial density, the initial inoculum density seems to be species dependent since cucumber required a higher inoculum density than cassava (Bergervoet *et al.*, 1989), while soy bean required a lower inoculum density for both initiation and maintenance (Finer and Nagasawa, 1988).

For suspension establishment, cassava cultures seemed to require more frequent subculture (every 5 days) for the first two weeks. This was in accord with the work of Vasil and Vasil (1980) showing that with *Pennisetum americanum* the cultures should be subcultured every two or three days for a period of two weeks. The subculture interval, once the suspensions have been established, seems to depend upon the plant species. Cassava embryogenic suspension cultures seemed to require a two- week subculture regime (Section 5.3.1.1), while those of the Gramineae required to be subcultured every week (Vasil and Vasil, 1980) and the majority of other plant species seem to require two

to four week subculture intervals (Hildebrandt, 1973; Gamborg *et al.*, 1983). These values, of course, depend also on the ratio of inoculum size to volume of medium employed in the various investigations.

Results (Section 5.3.1.3) indicated that a more uniform somatic embryo population could be obtained by sieving the initial heterogenous cell population. A physical method for the separation of various stages in the embryogenic cell suspension cultures has been reported by other investigators. Warren and Fowler (1977) used a bed of glass beads to separate various stages of embryogenic carrot suspension cultures and sieving followed by centrifugation in Ficoll solutions to synchronize carrot suspension cultures (Fujimura and Komamine, 1988).

The failure to induce division in the single cells isolated from the cassava leaves was perhaps not suprising in view of the difficulties encountered in obtaining sufficient numbers to provide a relatively high inoculum density. Halperin (1967) pointed out that the failure of cells to reach their full embryogenic potentiality at low cell densities is possibly because of the rapid loss of cell metabolites into the environment. Work with soy bean (Schwenk, 1980) showed that cells divided only at populations of 1.5-5.0 x 10^5 cells ml^{-1}, while sweet potato cells at a density of 1.0-5.0 x 10^4 cells ml^{-1} underwent division (Bidney and Shepard, 1980). In comparison, with the cassava it was only possible to achieve inoculum densities in the range 2.0-6.0 x 10^2 cell ml^{-1}.

It is possible that other factors, such as the type and concentration of hormones added to culture medium, might have caused the failure of cassava single cell cultures to divide. Street *et al.*(1965); Bhatt and Mehta (1974) stated that special nutritional conditions such as auxins and coconut milk are necessary to induce divisions in isolated higher plant cells. Single cells of carrot differentiated to somatic embryos when they

were cultured in a medium containing 2,4-D (5 x 10^{-8} M) plus zeatin (10^{-6} M) and mannitol (0.2 M) for seven days (Nomura and Komamine, 1985); when added alone, auxin was claimed to be necessary for single cells to form embryogenic cell clusters, while cytokinin showed little effect on embryogenesis from single cells. Single cell cultures of some species seem to require additional compounds to grow. Bayley *et al.*(1972), for example, demonstrated that glutamine could alleviate the reduced-nitrogen requirement of soy bean cells.

Bhojwani and Razdan (1983) argued that the composition of medium and the initial plating cell density are inter-dependent. When cells are plated at a high density, a purely synthetic medium with a composition similar to that used for suspension cultures or callus cultures is generally satisfactory, and the culture requirements of cells become increasingly complex as the plating density is decreased. This population effect on cell division can be overcome by the addition to the medium of undefined factors such as coconut milk, casein hydrolysate and yeast extract. Stuart and Street (1971) pointed out that, when sycamore cells were plated at a low density, it was necessary to add cytokinin, gibberellic acid and amino acids to the medium that was otherwise satisfactory for callus cultures.

Genotype would seem to be responsible for the difficulty in isolating single cells of cassava particularly by mechanical methods. Vasil (1982) argued that the ease of obtaining single cells is affected by the genotype, and Rossini (1972) has remarked that the mechanical method of cell isolation is not universally applicable. This is because only mesophyll cells of plants in which parenchymatous tissue was loosely arranged (having few points of contact between their cells) could be succesfully isolated. Single cells of some plant species, therefore, can be easily isolated such as sweet potato (Bidney and Shepard, 1980), *Ipomoea quamoilit* (Bhatt and Mehta, 1974), soy bean

(Schwenk, 1980), while single cells of *Rauwolfia tetraphilla*, and *Samanea saman* (Gnanam and Kulandaivelu, 1969) are not readily isolated.

Like mechanical isolation, enzymatic isolation seems not to be universally applicable. Otsuki and Takebe (1969) showed that *Hordeum vulgare*, *Triticum vulgare*, and *Zea mays* were difficult subjects for cell isolation through enzymatic methods. They presumed that the reason for leaves of monocotyledons tending to be macerated more slowly than those of dicotyledons was partly due to the presence of mucilage in the leaves preventing access of the enzyme to its substrate. Both the rate of maceration and the yield of intact cells vary considerably depending on the age and physiological state of plants. Evans and Cooking (1975) argued that mesophyll cells of cereals appear elongated with a number of constrictions, and within the leaf these cells may form an interlocking structure preventing their isolation.

The addition of EDTA seemed to accelerate maceration and slightly increased the yield of cassava leaves (Section 5.3.2.2). This is in agreement with the work of Otsuki and Takebe (1965) indicating that EDTA helped to accelerate the maceration of the leaves of plants from which single cells are difficult to isolate.

The culture method might also have been responsible for the failure of the cassava single cells which were cultured either on semi- solid medium, liquid medium or two layers of medium (Section 5.3.2.2). Muir *et al.*(1958), in their pioneering work, suggested that single cells required a fairly moist surface for growth. They showed that single cells could only be grown successfully on filter paper over established cultures, while those cultured on agar medium, on filter paper over agar medium or in sealed hanging drops of acclimated medium were unsuccessful.

The results showed that an 18 hour incubation in enzyme solution was required to obtain the optimum yield of cassava protoplast from somatic embryos (Section 5.3.3). It seems that the optimum duration of digestion, depending on the type and composition of enzyme used, is genotype dependent. Rubos (1985), for example, proved that 24 hour incubation period was required for the release of protoplasts from carrot embryos.

Like single cells, the growth and division of protoplasts seems to be affected by plating density. Protoplasts plated at a density of 2.0-2.8 x 10^5 protoplasts ml^{-1} showed a better growth (Section 5.3.3.2). Protoplasts of different plant species seems to require different plating densities. Protoplasts of pea were successfully plated at a density of 5.0 x 10^4 protoplasts ml^{-1} (Jia, 1982), while those of *Larix x eurolepis* were plated at a density of 1.0 x 10^5 protoplasts ml^{-1} (Klimaszewska, 1989), and those of wheat were plated at a density of 1.5-2.0 x 10^5 protoplasts ml^{-1} (Harris *et al.*, 1988).

The failure of protoplasts to undergo cell division at a higher frequency and to form callus (Section 5.3.3) was possibly caused by the plating method, since the protoplasts incubated in small drops as opposed to those in liquid or semi-solid medium covering the whole dish had a higher incidence of survival and cell division. The advantage of culturing protoplast in a small drops according to Bawa and Torrey (1971), and Farmer and Lee (1977) could be due to the difference in the amount of movement within the medium when handling in Petri dishes, which could affect the breakage or the amount of clumping of the protoplast. Clumping of the protoplasts creates a high density zone with a lack of nutrients and a high concentration of toxic materials released by the dead cells which could lead to a lower survival rate.

The composition of medium may have been responsible for the failure of protoplasts to undergo further growth (Section 5.3.3). A more complicated medium such as medium

with the addition of extra reduced nitrogen or vitamins is possibly required for culturing protoplasts from cassava somatic embryos. Bui-Dang-Ha and Mackenzie (1973) demonstrated that glutamine at a level of 1000 mgl^{-1} added into the growth medium was the key to the success of culturing protoplasts of asparagus. Bhojwani and Razdan (1983) believed that the type of auxin and cytokinin and their ratios in the medium required for optimal growth may vary considerably with the plant material. In tobacco, NAA was superior to 2,4-D or IAA for the culture of protoplasts from cell suspensions (Uchimiya and Murashige, 1976), whereas those of soy bean required NAA and 2,4-D used together in combination with BAP (Wei and Xu, 1988).

The further possible cause of the protoplasts ceasing to divide might have been that the addition of fresh medium at a lower osmolarity was too late (Section 5.3.3). Bhojwani and Razdan (1983) pointed out that the addition of a few drops of fresh medium lacking the osmoticum was needed when the initial culture was 7-10 days old by which time most of the viable protoplasts had regenerated a good wall and undergone a few division. In protoplast cultures of *Larix x eurolepis* (Klimaszewska, 1989), the addition of fresh medium was carried out after 10-12 days in culture, while pea protoplasts (Jia, 1982) required earlier fresh medium addition (2 days after isolation) to increase the division.

CHAPTER 6

ANATOMY AND MORPHOLOGY OF SOMATIC EMBRYOS

6.1. INTRODUCTION

The question of whether somatic embryos originate from single cells or from groups of cells has aroused interest because of implications concerning the morphogenetic relationship between somatic and zygotic embryos. It is also of practical significance, particularly where embryogenic systems are used for the regeneration of transgenic plants which are more likely to have a chimeral structure if they are of multicellular origin.

Using both light and electron microscopy, a number of investigators have attempted to follow the ontogeny of a somatic embryo from single cells during both direct and indirect somatic embryogenesis but the destructive nature of these methods usually presents unequivocal conclusion from being drawn. Konar *et al.*(1972) demonstrated the likely formation of somatic embryos of *Ranunculus sceleratus* from single epidermal cells of the stem of *in vitro* plantlets. The apparent single cell origin of somatic embryos has also been demonstrated using other plant cultures such as *Daucus carota* (McWilliam *et al.*, 1974), *Nigelia sativa* (Banerjee and Gupta, 1976), sugarcane (Nadar *et al.*, 1978), *Brassica oleraceae* (Pareek and Chandra, 1978), celery (Dunstan *et al.*, 1982). On the other hand, Constabel *et al.*, 1971; Sussex, 1972; Litz and Conover, 1983 have demonstrated that somatic embryos arose as multicellular budding from epidermal cells or from embryogenic aggregates in culture systems. There is also evidence that the two origins could occur concurrently in the same culture system. Dunstan *et al.*(1978) reported that somatic embryos of *Sorghum bicolor* cultured *in vitro* were formed directly on the cultured embryos in which case the scutellum of the secondary embryos arose by folding of the primary scutellum without *de novo* formation, while others appeared to arise from single cells of the primary scutellum. Maheswaran and Williams (1985) have produced convincing evidence from both light and electron microscope studies of

Trifolium repens cultures that multicellular budding and single cell initiation of somatic embryos both occur from the epidermis of the immature embryos.

6.2. MATERIALS AND METHODS

6.2.1. Plant materials

Primary somatic embryos of different ages attached to leaf lobes surface, clumps bearing somatic embryos cultured in liquid medium, and secondary somatic embryos were used for sectioning and scanning electron microscope studies using the techniques described in Materials and Methods (Chapter 2). All materials had been subjected to medium supplemented with either 2.0 or 4.0 mgl^{-1} 2,4-D.

6.2.2. Sample preparations for light microscopy

The difference between primary somatic embryos, secondary somatic and non embryogenic tissues were compared using both a squash preparation and sectioning procedure. Somatic embryos and non embryogenic tissues were squashed on a glass slide and observed under the light microscope using a polarizing filter. The details of sectioning procedures are described in Chapter 2.

6.3. RESULTS

Observations suggested that primary and secondary somatic embryos developed via similar developmental sequences (Plates 6.1-6.3). Secondary somatic embryos were morphologically identical to primary embryos and responded with further somatic embryogenesis or plantlet development when cultured under appropriate conditions.

There was a relationship between the size of explant and callus and embryogenic-tissue initiation. Most cotyledons of primary embryos and leaf lobe explants in the range of size of 1-3 mm initiated embryogenic tissues only, while the larger sizes of explant were always associated with friable callus formation. The number of somatic embryos produced by 1-3 mm leaf lobes, however, was lower than that produced by 3-5 mm leaf lobes. In the case of primary somatic embryos, the embryogenic tissues were most likely to be initiated on the adaxial surface area near to the midvein.

Sections of embryogenic tissue at 13 days showed protuberances which displayed special features making it possible to distinguish them from non-embryogenic callus and to consider them as embryogenic structures (Plate 6.1A).

The difference between friable callus and somatic embryos could also be observed by squash preparations viewed with polarized light. It seemed that somatic embryos, in particular the mature ones, characteristically contained crystals which were identified as calcium oxalate in the middle of hypocotyl. This type of crystal was absent from friable callus.

Plate 6.1B shows a longitudinal section through a young somatic embryo attached to the adaxial surface of a leaf lobe explant cultured for 25-32 days (14 days in Stage-I medium and 11-18 days in Stage-II medium). It was noted that the cotyledon initiation and the shoot meristem of this embryo were clearly visible and the position of development of vascular tissues connecting the root axis region to the cotyledons was indicated by more densely staining cells. The further development of somatic embryos which was indicated by the production of well defined cotyledons, vascular tissues and root and shoot axes, took place when the leaf-lobe cultures were finally transferred to hormone-free medium.

Microscopial observations also showed that the shoot axis of the somatic embryos became determined before the root axis. The cotyledons of somatic embryos became green and enlarged after 24-30 days in culture and were attached to a hypocotyl which tapered into the parental tissues. Like primary somatic embryos, secondary embryos which developed exclusively from the shoot axis and cotyledons of the primary embryos, also possessed closed radicular ends and there were no vascular connections with the parental tissues (Plates 6.1C,D). Scanning electron micrographs also revealed that the surfaces of secondary embryos were composed of cells of similar types to those found in primary embryos (Plate 6.2).

Sections of clumps bearing somatic embryos (Plate 6.5) showed that somatic embryos formed in shaken liquid medium were histologically similar to those cultured on semi solid medium. It was noted, however, that those cultured in liquid medium were composed of smaller and more dense cells, and root formation occured more frequently.

The highly embryogenic suspension cultures were composed of embryogenic clumps and of vacuolated free cells and small aggregates of such cells. The vacuolated

free cells probably arose by being released as the proliferating embryogenic clumps fragmented. The superficial cells were densely cytoplasmic and they had a large diffusely-staining nucleus with a single prominent darkly-staining nucleolus. Somatic embryos were rapidly initiated from the superficial cells of the aggregates and when the cultures were transferred to medium lacking 2,4-D these aggregates became studded with asynchronously developing somatic embryos.

Somatic embryos normally developed as isolated structures with two cotyledons but some with fused cotyledons or with more than two cotyledons were also noted (Plate 6.5). These abnormalities other than the fused cotyledon did not appear to affect the developmental potential of the embryos under appropriate conditions. The growth of embryos with fused cotyledon seemed to be very slow and frequently they only produced roots, unless the cotyledon was removed manually.

Further proliferation of somatic embryos was normally caused by the growth of new embryos from the base of existing embryos. However, it was noted that new somatic embryos frequently emerged from the hypocotyl by a "budding" process (Plate 4.2A). The budding process continued when the clumps of somatic embryos were transferred to further medium containing 2.0 or 4.0 mgl^{-1} 2,4-D after the removal of non embryogenic tissues.

Observations of the regeneration process revealed that the growth of secondary embryos was affected by the shape of the secondary tissue initially produced (Plate 4.1). Various protuberances and ridges could be observed in 15 day old cultures, including circular, cylindrical, eliptical and heart shaped structures. At day 25, it was observed that approximately 9% of the secondary tissues were heart shaped, 15% were of cylindrical shape, 31% were of circular shape and 44% were of elliptical shape. Out of the shapes

observed, the cotyledons could only be regenerated from the tissues of circular and elliptical shape. The elliptically shaped tissues, however, seemed to grow more quickly and to produce a higher frequency of mature somatic embryos possessing cotyledons following transfer to medium lacking 2,4-D. It was also noted that a single tap root emerged from embryos produced from this type of tissue.

Plate 6.1.

Sections of primary and secondary somatic embryos stained with Toluidine blue.

A. Longitudinal section of leaf-lobe explant showing the formation of embryogenic tissue after 13 days. x125.

B. Longitudinal section of leaf-lobe explant showing the formation of primary somatic embryos after 25 days. Note the developed cotyledons possessed by somatic embryos. x47

C and D. Longitudinal sections of primary somatic embryos showing the formation of secondary somatic embryos. Note that there is no vascular connection to parental tissue. x41.

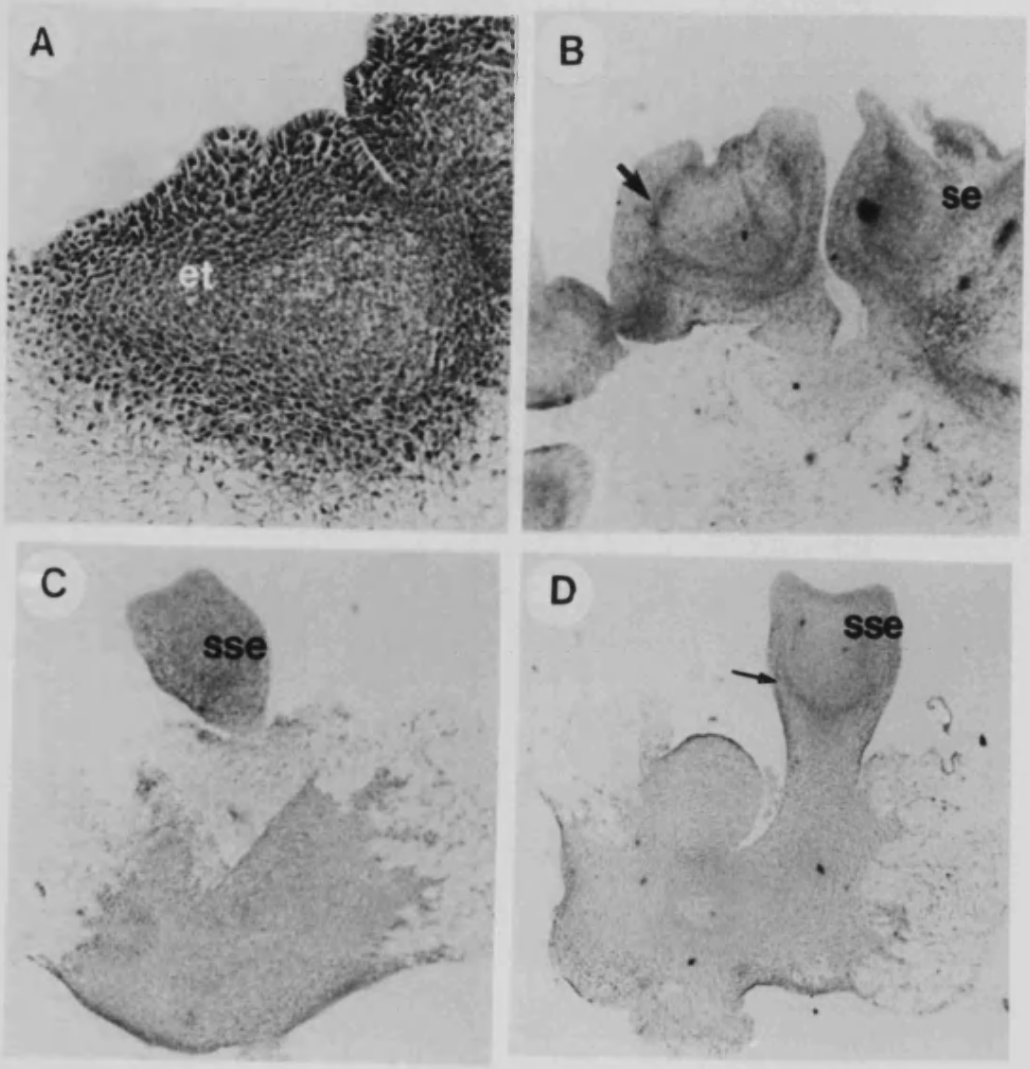
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Plate 6.2.

Scanning electron micrographs of primary and somatic embryos

A. Scanning electron micrograph of early primary somatic embryos (pet) attached to leaf-lobe explant (llb) Bar represents = 100 µm.

B. Scanning electron micrograph of secondary somatic embryos (sse) attached to the cotyledon of primary embryo (ct). Bar represents= 100 µm.

C. Scanning electron micrograph of secondary somatic embryos Bar represents = 100 µm.

D. Scanning electron micrograph showing the formation of secondary embryos from the base of a primary embryo. Bar represents = 100 µm.

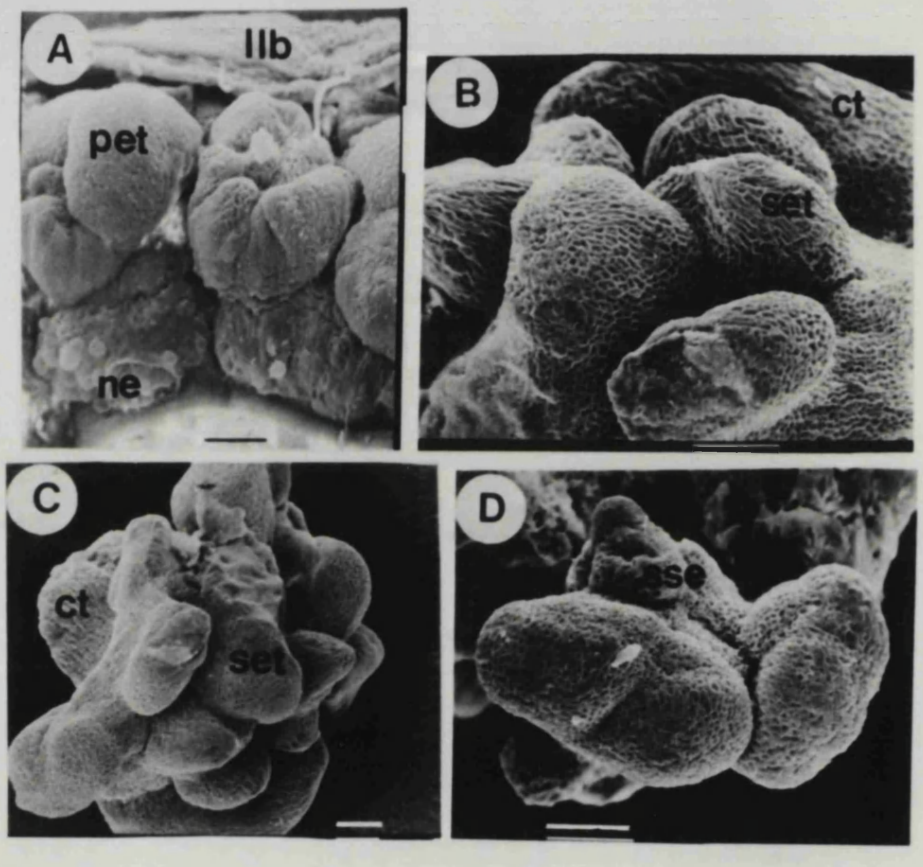
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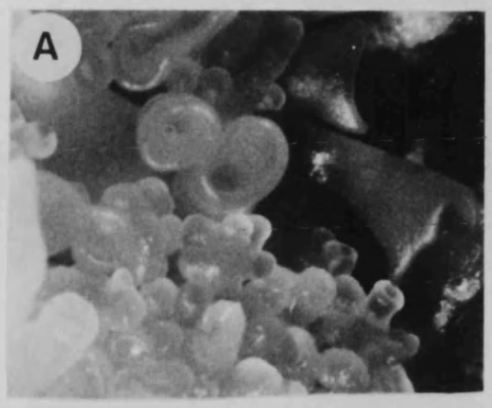
Plate 6.3.

The induction and regeneration of secondary somatic embryos.

A. Extensive proliferation of secondary somatic embryos on medium supplemented with 4.0 mgl^{-1} 2,4-D. x29

B. Scanning electron micrograph showing later stage of secondary somatic embryos.

C. Regeneration of secondary somatic embryos on hormone-free medium. x25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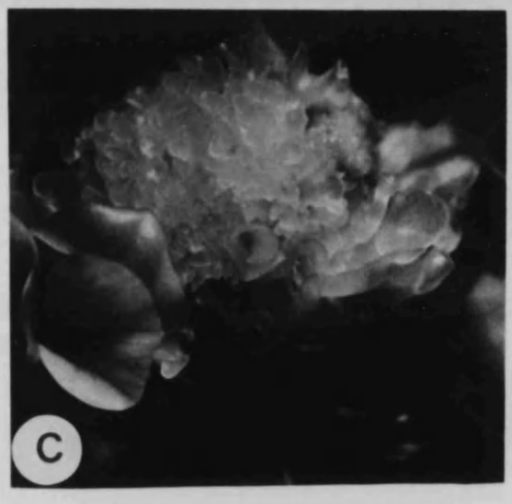
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Plate 6.4.

Regeneration of abnormal somatic embryos showing fused cotyledons.

A. Two primary somatic embryos showing tubular cotyledons (arrow). x25

B. Scanning electron micrograph showing primary somatic embryos with fused cotyledons. Bar represents = 100 μm.

C. Longitudinal section of primary somatic embryo with fused cotyledons. x25.

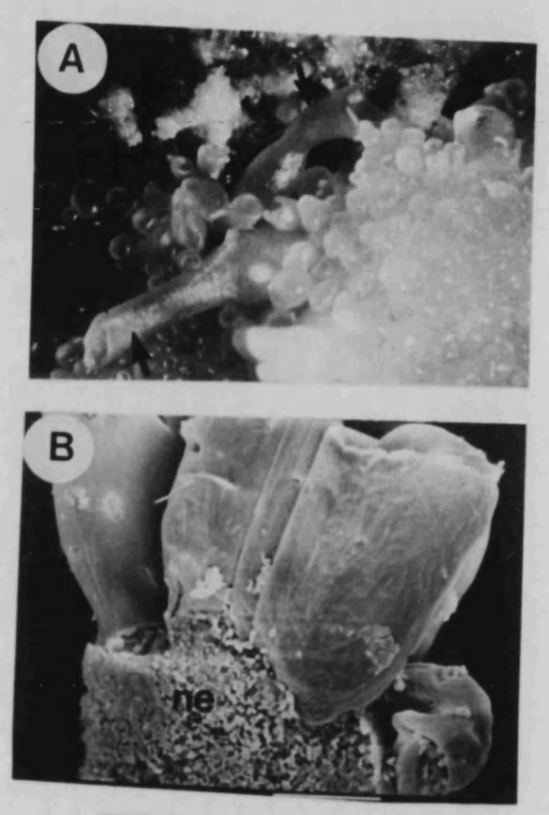
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Plate 6.5.

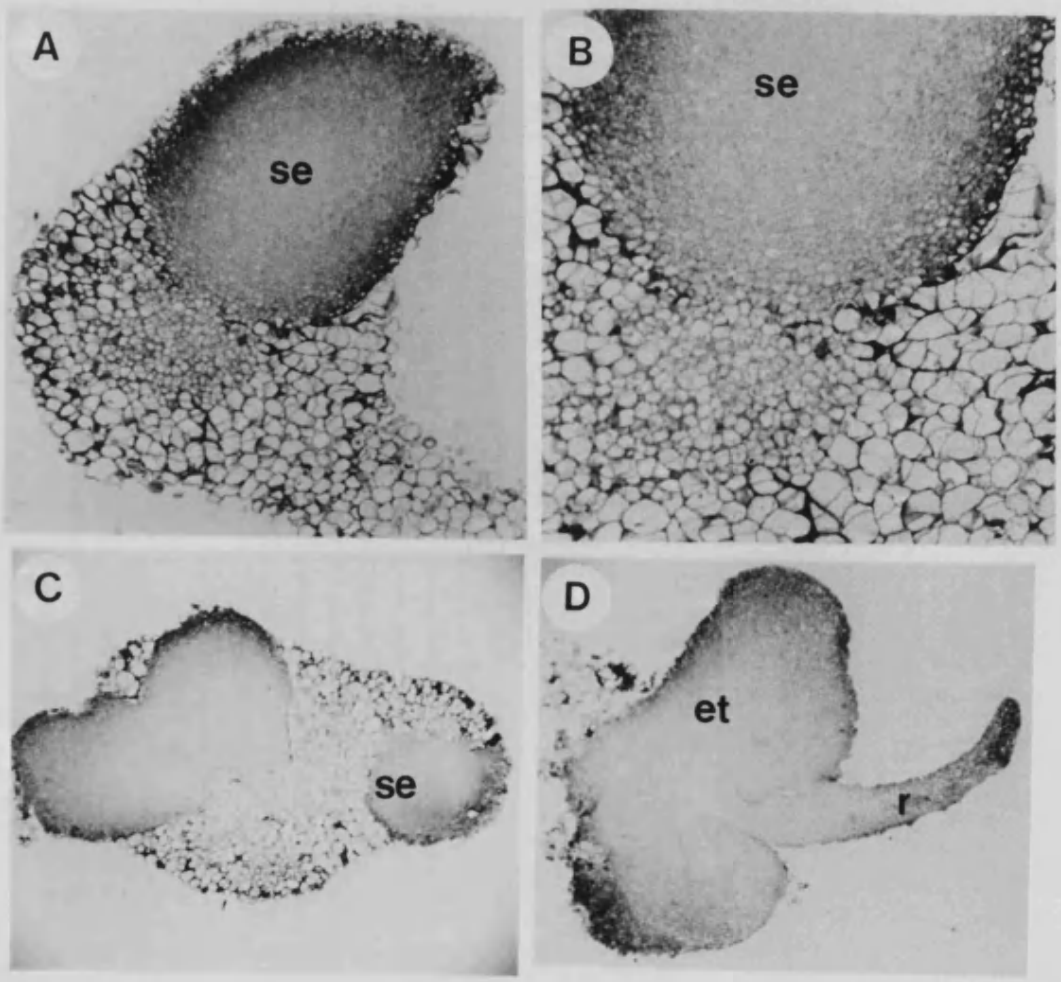
Sections of clumps bearing somatic embryos formed in suspension cultures.

A. Longitudinal section through embryogenic clump showing early stage of somatic embryo formation. x56

B. Same as A at higher magnification. x87.5

C. Longitudinal section through clump bearing somatic embryos. x28

D. Longitudinal section through an embryogenic clump bearing a root. x28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6.4. DISCUSSION

Results indicated that both primary somatic embryos growing from both semi solid and liquid medium and secondary somatic embryos were composed of small cells with dense cytoplasm and small vacuoles (Plates 6.1 and 6.6). Sharp *et al.*(1980), Bhojwani and Razdan (1983), Karlson and Vasil (1986) categorized embryogenic cells as small with nucleolus, dense cytoplasm and small vacuoles accompanied by a considerable accumulation of starch. Guiderdoni and Demarly (1988) categorized cells in sugarcane culture into three distinct types of cells : (a) epidermis cells with a high nucleocytoplasmic ratio, (b) small-sized meristematic cells with high nucleocytoplasmic ratio, dense cytoplasm and thickened cell walls, (c) larger-sized subepidermic cells possessing rich cytoplasm and containing conspicuous starch grains. These supported the observations of Thomas *et al.*(1972) in work with *Ranunculus sceleratus* that somatic embryo cells were categorized into two cells namely superficial cells which are highly cytoplasmic and inner cells which are larger, with less dense cytoplasm and more vacuoles. Halperin and Jensen (1967) pointed out that the inner or central region might contain a few large starch grains or no starch at all depending upon the age of the culture and the individual clumps.

The observations Konar *et al* (1972) showed that somatic embryos were formed as a result of the organized divisions in the superficial highly cytoplasmic cells which led to the formation of small groups of cells. Transfer of an embryogenic culture to medium lacking 2,4-D led to a rapid initiation of somatic embryos from the superficial cells of the aggregates and these were later released in the liquid medium (Street and Withers, 1974). Fragmentation of clumps occurred when the older cells at the centre of clumps became separated from each other. The mechanical effects in rotating liquid medium undoubtedly hasten the fragmentation. The smaller clumps released from a parent

clump are clearly polarized by virtue of the manner in which they form. Each daughter clump bears large vacuolate cells at one end and smaller meristematic cells at the other (Halperin and Jensen, 1967). In young leaves of sugarcane (Guiderdoni and Demarly, 1988), cells started to divide within vascular parenchyma near the phloem pole of vascular bundles. Cellular divisions spread to the other tissues (mesophyll and epidermis) and were more intensive towards the abaxial surface.

The histological observations also showed that cassava somatic embryos cultured in solid and liquid medium were similar with respect to the absence of vascular connections with the parental tissues. A suspensor was not observed and the hypocotyl of somatic embryos tapered into the parental tissues. This is in agreement with Ho and Vasil (1983) in work with sugarcane showing that somatic embryos appeared to lack a suspensor structure; the proembryos and somatic embryos were attached to the underlying tissue by a suspensor which was either indistinguishable or possibly present as a broad multicellular structure. Trigiano *et al.*(1988) observed that somatic embryos were formed without an intervening callus and were attached directly to the parental tissues by a bulbous, suspensor-like structure that lacked vascular tissue.

It would seem that both direct and indirect embryogenesis were observed in embryogenesis of cassava. Most older leaf-lobe explants produced a densely-packed callus before the somatic embryos formed on the top of callus. This pattern of embryogenesis usually led to a relatively low number of somatic embryos being produced per explant. By contrast, secondary somatic embryos were formed directly from primary embryos without the intervenion of a callus stage and in greater numbers. Pence *et al.*(1980) observed that direct embryogenesis in *Theobroma cacao* was characterized by hypocotylary budding; this direct embryogenesis gave rise to four-cell globular bodies which were connected to the hypocotyl via suspensor-like structures

consisting of 3-4 cells. In indirect embryogenesis in this species, according to Kononowicz *et al.* (1984), the initial precursor and the very earliest stage of somatic embryo formation could not be absolutely identified and the suspensor-like structures could not be recognized until the globular stage of development.

The results show that some abnormalities in terms of the shape and number of cotyledons was observed in the cassava somatic embryos (Section 7.4). The abnormal somatic embryos, however, were still able to regenerate to plantlets, except of those with the fused- tubular shaped cotyledons. This in agreement with Litz and Conover (1983) in work with *Carica papaya* showing that normal plantlets develop from somatic embryos possessing an unusual number of cotyledons. Abnormal somatic embryos were also observed in *Poa pratensis* L (van der Valk *et al.*, 1989) and of *Hevea brasiliensis* (Michaux - Ferriere and Carron, 1989). Scanning electron micrographs of somatic embryos of *Poa pratensis* showed many multiple embryos with fused scutella and leafy scutella. The failure of fused tubular cotyledon of cassava somatic embryos to regenerate into plantlets was apparently caused by poor individualization of the shoot meristem between cotyledons which developed unequally. Histological preparations of somatic embryos of *Glycine max* (L)Merr (Trigiano *et al.*, 1988) demonstrated that the shoot apices were broadly flattened and enlarged compared to the dome-shaped shoot apices in fully-developed zygotic embryos. This suggested that shoot apices of some somatic embryos differentiated prematurely and possibly would not produce shoots during germination (Ammirato, 1987).

CHAPTER 7

PRODUCTION OF PLANTS

7.1. INTRODUCTION

7.1.1. Plantlet regeneration

Regeneration of plantlets via somatic embryogenesis is, in principle, the most efficient approach for true clonal multiplication and biotechnological applications (Vasil, 1982) provided that major problems associated with genetic stability and synchrony of embryogenesis can be solved. Therefore, an efficient production of normal plantlets possessing both shoot and roots is necessary to assist the application of biotechnology toward the genetic modification and improvement of important group of crop plants. It is well known,however, that one of the problems associated with the process of somatic embryogenesis is the abnormal nature of some of plantlets that are produced. Failure of somatic embryos to produce a shoot with normal leaves and stem most likely can be attributed to the malformation of the shoot apical meristem. In this case, the somatic embryos may look normal in terms of their external morphology but may be abnormal in terms of cellular and tissue differentiation (Kerns *et al.*, 1986; Ammirato, 1987).

Several investigators have reported that the abnormality of somatic embryos could be reduced by using certain treatments. In *Carum carvi*, the addition of 0.1-1.0 μmoll^{-1} ABA to the medium has been shown to normalize the development of somatic embryos by inhibiting abnormal proliferation, the development of adventitious embryos along the hypocotyl, the formation of multiple and abnormal cotyledons and precocious germination (Ammirato, 1974). ABA has been shown to modify the formation of all abberant forms of somatic embryos (Vasil and Vasil, 1981, 1982; Ranch *et al.*, 1985).

Other hormones such as GA_3 have also been as an aid to somatic embryo maturation (Ammirato, 1982; Lu *et al.*, 1982); for example, application of GA_3 is

required for root and shoot development during germination of somatic embryos in *Citrus sinensis* (Kochba *et al.*, 1972).

In some species a cold treatment of young or mature somatic embryos is required for their normal development. In grapes, chilling the cultures bearing somatic embryos at any stage from globular to maturity at 4°C for two weeks was essential to stimulate the formation of normal plantlets (Rajasekaran and Mullins, 1979). Similarly, somatic embryos of *Eschscholtzia californica* must be exposed to a low temperature to enable them to germinate (Kavathekar *et al.*, 1977).

Normal plantlets, usually need to be hardened before they can be transferred to greenhouse conditions, otherwise they undergo desiccation and death when they are transferred to the less humid external environment. It is a general observation that the high humidity of the environment *in vitro* does not allow the synthesis of cuticlar and epicuticular wax on the epidermis of leaves of regenerated plants (Sutter, 1985). Also, Brainerd and Fuchigami (1982) pointed out that the stomata of plants *in vitro* do not seem to have closure mechanism which the main cause of rapid loss during transfer to low relative humidity. Therefore, the acclimitization procedure is required for such cultured plants during which they both develop cuticles and mechanisms controlling the closure of stomata.

7.1.2. Genetic stability of somatic embryos and plantlets

Reports about the genetic stability of somatic embryos and plants derived from somatic embryos are contradictory. Krikorian (1982) reported that the phenotypes of *Daucus carota* plants raised from somatic embryos were normal, provided that the

suspensions from which they were arrived were maintained for relatively short periods such as less than one year. Also, Schuller *et.al.* (1989) demonstrated that plants derived from *Abies alba* somatic embryos were diploid (2n=24).

Other investigators believe that the frequency of abnormalities, such as changes in karyotype would be likely to increase with increasing time in culture. Variability in chromosome number (Takatori and Murashige, 1968; Roberts, 1975) or changes in karyotype structure (Bayliss, 1980; Krikorian *et.al.*, 1981; Flashman, 1982) were demonstrated in long-term cultures. Work with oat cultivars Lodi and Tippecanoe showed that the frequency of cytogenetically abnormal plants increased with culture age: abnormal plant from young cultures usually possessed single alterations, whereas abnormal plants from cultures up to 20 month old frequently had more than one chromosome alteration (Mc. Coy, 1980). By contrast, long-term cultures of corn (Mc. Coy, *loc.cit.)* were more stable as 94% of the plants analyzed after 8 months were normal.

Variability in chromosome number (Takatori and Murashige, 1968; Roberts, 1975) or change in karyotypic structure (Bayliss, 1980; Krikorian *et.al.*, 1981; Flashman, 1982) have been proved as a result of long term cultures. Changes in chromosome karyotype have been observed in somatic embryos and plants of *Bromus inermis* (Gamborg *et.al.*, 1970), *Lolium multiflorum* (Dale, 1980), and *Hemero callis* (Krikorian *et.al.*, 1980) which resulted in more than 50% albino. Loss of embryogenic potential which brought about the decrease in embryogenic capacity of cultures decreased and disappeared during progressive subculturing (Syono, 1965) was apparently caused by the change in chromosome complement where aneuploids gradually replace diploid cells (Torrey, 1967; Smith and Street, 1974). Larkin and Scowcroft (1981) have pointed out that gross chromosomal changes and abnormalities were not the only, or even the main

causes of what they have called "somaclonal variation". It has been suggested that the frequent establishment of fresh cultures from plant materials and careful attention to the subculture regime may help maintain genetic and chromosomal stability.

7.1.2.1. Cytology and cytological techniques

Chromosomes counts of regenerated plants arising either via organogenesis or somatic embryogenesis are usually obtained from metaphase cells of root tips. Krikorian *et.al.*(1982) state that counting of metaphase chromosomes in squash preparations is the most reliable method of determining the chromosomes number and degree of ploidy of the plant. Counting is, however, subject to error if membranes are destroyed or broken, so that the spread chromosomes are likely to intermix. Root-tip squashes might also not be satisfactory or even possible under all conditions; for example, when a seed was shrivelled and germinated slowly with one or two small roots, or when a seedling was weak at the time of transplanting, chromosomes counts were not possible (Sharma and Gill, 1984).

7.1.2.2. Cytology and cytological techniques with cassava

In all of the species of the tribe Manihoteae the 2n chromosome number had been found to be 36 in root tips and 18 in microsporogenesis (Graner, 1941; Perry, 1943; Capinpin and Bruce, 1955). Perry (1943) also pointed out that in the vast majority of cases, chromosomes paired as bivalents.

A squash procedure was adopted for counting the number of chromosomes in the root tips. Capinpin and Bruce (1955) used a fresh mixture of 2.5 parts of 3% ferric ammonium sulphate and 7 parts of 95% alcohol applied for three hours or overnight following fixation in 1:3 acetic-alcohol for one half to three hours. Sohmer (1968) used Carnoy's fixative (3 parts of absolute alcohol, 1 part of acetic acid and 2 parts of chloroform) for a period of 12-24 hours. In both cases, staining was carried out with aceto carmine (Capinpin and Bruce, 1955; Sohmer, 1968).

7.2. MATERIALS AND METHODS

7.2.1. Plantlet regeneration

All somatic embryos had been cultured for 14 days on MS medium containing 4 mgl^{-1} 2,4-D and for another 30 days on hormone-free MS medium.

Unless otherwise stated, the medium used for inducing roots and the hardening of the plantlets was hormone-free half-strength MS medium.

Somatic embryos bearing shoot were placed on either filter bridges or 'Milcap' plugs (Milcap, France) in test tubes (24 x 150 mm). Twyford boxes 5.2 x 3.4 inch in size were used to harden plantlets *in vitro*, while mini propagators 8 x 6 inch in size were used to harden those *in vivo*.

Finally plantlets were transferred to 3.5 inch diameter pots containing Levington compost, and they were placed in the green house on sand benches.

7.2.2. Chromosome counting

Root tips 2-4 mm in length taken from plants regenerated from somatic embryos growing *in vitro* and somatic embryos taken from clumps of somatic embryos growing on media supplemented with 2,4-D were used for genetic stability studies.

Flat bottom tubes soda glass with polythene closures, 50 x 19 mm in size (Samco) were used to contain the root tips during pretreatment, fixation, storage, hydrolysis and staining.

Root tips or somatic embryos were placed in the 50 x 19 mm flat-bottomed tubes containing pretreatment solution and incubated for two to five hours. After removing the pretreatment solution, the fixative was poured in the tubes. Having removed the fixative solution and rinsed the tissues with distilled water, 1N HCl was poured in the tubes. They were then placed in water bath set at 60^{o}C for 10-30 minutes. Staining was done after HCL was removed. Depending on the stain solution used, the samples were either kept in the dark or under the light. Root tips were placed on a glass slide and the brownish meristem regions 1-2 mm in length were cut out with a blade. The individual root tips in afew drops of stain solution were gently tapped with the wooden end of a dissecting needle. After placing the cover slips, they were gently heated over the flame of an alcohol burner. Having placed them in between a folded filter paper, they were squashed vertically using thumbs. The same procedure was used to squash the somatic embryos. The flattened tissues were viewed under an Olympus light microscope.

7.3. RESULTS

The experiments were categorised into three major sections : investigations to improve the production of normal plantlets, investigations to increase the survival following transfer to the greenhouse, and investigations to check the genetic stability of somatic embryos and of plantlets regenerated from somatic embryos.

7.3.1. The effect of the size and nature of individual somatic embryos on plantlet development

Primary somatic embryos in four different ranges of size (1-3, 3-5, 5-7, and larger than 7 mm) and secondary embryos (1-3 mm in size) were cultured individually on regeneration medium. The frequency of normal plantlets regenerated from these embryos was scored and displayed in Tables 7.1 and 7.2.

The 1-3 mm primary embryos possessing two cotyledons gave rise to the highest frequency of normal plantlet production (16.44%). It seemed that the larger the size of somatic embryos, the lower the frequency of normal plantlets that could be obtained. Plate 7.1A,B show the regeneration of normal shoot from clumps of somatic embryos.

Secondary embryos seemed to be superior to primary embryos in terms of the production of normal plantlets, with the frequency being 84.4% and 33.3% respectively (Table 7.2).

The frequency of normal plantlet production was also apparently affected by the shape of the somatic embryo cotyledons. Somatic embryos with fused, tubular

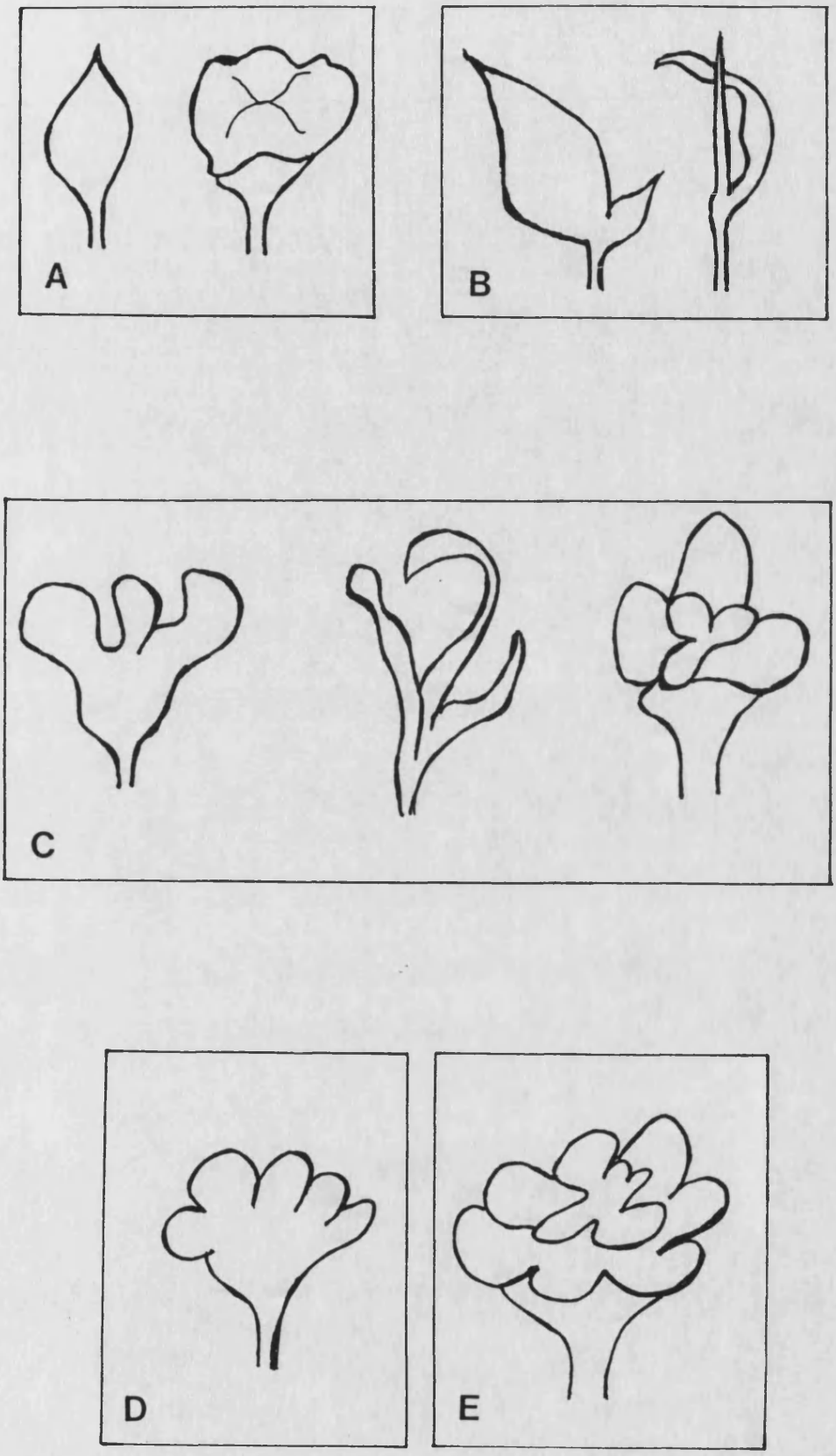


Fig. 5.1. Types of abnormal somatic embryos

Table 7.1. The effect of the size of somatic embryos on the production of normal plantlets

Size of somatic embryos (mm)	% somatic embryos converting to normal plantlets
1-3	16.4
3-5	7.1
5-7	3.7
>7	3.3

Table 7.2. The effect of the nature of somatic embryos on the production of normal plantlets

Nature of somatic embryos	%somatic embryos converting to normal plantlets
Primary embryos	33.3
Secondary embryos	84.4

Key

Number of replicates : 10

Regeneration medium : hormone-free MS medium supplemented 2% sucrose

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, $30 \mu Mm^{-2}s^{-1}$ PAR

cotyledons, irrespective the sizes tested, did not grow further, while those possessing two or more cotyledons could regenerate to normal plantlets as long as they were 1-3 mm in size. Normal plantlets, however, could be regenerated from the tubular embryos if they were cultured on regeneration medium for a longer period (two months) during which time the fused cotyledons dried and detached from the hypocotyl. To accelerate this process the fused cotyledons could be removed manually from the hypocotyl. Plate 7.1D. shows plant regeneration from a tubular somatic embryo.

7.3.2. The effect of ABA and GA_3 on plantlet development

ABA (0.2, 2.0 or 4.0 mgl^{-1}), GA_3 (2.0 mgl^{-1}), and activated charcoal (1.0 gl^{-1}) were added to the medium for culturing clumps of somatic embryos for one month. The embryos were then separated and transferred individually to regeneration medium. After one month on this medium, the frequency of normal plantlets was scored as shown in Table 7.3.

The highest frequency of normal plantlets (12.5%) was obtained by culturing clumps of somatic embryos on medium supplemented with 2 mgl^{-1} ABA prior to culture on regeneration medium. A higher level of ABA (4 mgl^{-1}) seemed to make the clumps of somatic embryos water-logged and they lost their morphogenic potential if they were left for a longer period.

The addition of GA_3 to the medium seemed to accelerate the growth of somatic embryos resulting in 60% of clumps of somatic embryos possessing large cotyledons (larger than 7 mm). The frequency of normal plantlets obtained from this medium, however, was only 10% (Table 7.3).

Table 7.3. Regeneration of clumps of somatic embryos that had been maintained on medium supplemented with either ABA, GA_3 or activated charcoal

Media			% clumps possessing large cotyledons	%mature embryos producing roots only	% mature embryos converting to normal plantlets
activated charcoal (gl^{-1})	ABA (mgl^{-1})	GA^3 (mgl^{-1})			
-	-	-	39.2	28.6	5.4
1.0	-	-	56.7	38.9	1.1
-	-	2.0	60.0	6.7	10.0
-	0.2	-	22.7	13.6	9.1
-	2.0	-	6.3	6.3	12.5
-	4.0	-	0	1.3	0
-	4.0	2.0	5.0	1.3	2.5

Key

Number of replicates : 10

Medium : hormone-free MS medium supplemented with 2% sucrose

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$PAR

7.3.3. The induction of roots on mature somatic embryos

It frequently happened that the somatic embryos only regenerated shoots without root formation. Four rooting experiments (either on solid or liquid medium) were carried out to investigate the induction of the tap root and vigorous adventitious roots.

7.3.3.1. The induction of tap roots from plantlets regenerated from somatic embryos

Unlike plantlets regenerated from secondary embryos, those regenerated from primary embryos did not seem to possess a tap root. Plantlets possessing root initial were placed on 'Milcap' plugs in 24 x 150 mm test tubes and saturated with 10 ml of either quarter or half strength MS liquid medium (Plate 7.1E) The frequency of plantlets forming a tap root is displayed in Table 7.4.

Plantlets regenerated from somatic embryos growing on quarter-strength medium did not show further growth, while those on half-strength medium grew as indicated by the development of the tap root. The frequency of normal plantlets possessing a tap root, however, was still very low (20%). This frequency could be increased to 80% by culturing individual secondary somatic embryos on semi-solid hormone-free MS liquid medium which naturally produced a tap root (Plate 7.1C).

Table 7.4. Induction of tap root of plantlets placed on 'Milcap' plugs saturated with different strength of MS liquid medium

Medium	% plantlets producing top root
quarter strength	0
half strength	20

Key

Number of replicates : 10

Basal medium : MS (strength as indicated)

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, $30\mu Mm^{-2}s^{-1}$PAR

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D medium, 21 days on hormone-free medium, and a further 14 days on hormone-free medium after somatic embryos were separated individually

7.3.3.2. The effect of GA_3 on adventitious root induction

The effect of GA_3 (1 mgl^{-1}) added to both semi solid and liquid hormone-free MS medium, on the induction of adventitious roots was studied. Somatic embryos had been cultured on hormone-free MS medium were separated individually and cultured on medium containing GA_3. After 30 days, the number of individual mature embryos possessing roots was scored.

It seemed that the effect of GA_3 on adventitious root induction was affected by the state of medium. On semi-solid medium, the frequency of rooting increased from 6.7% to 17.6% if the medium was supplemented with GA_3. The highest frequency of rooting (28.6%), however, was obtained with somatic embryos cultured in liquid medium devoid of GA_3. The addition of GA_3 in liquid medium decreased this frequency to 14.3% (Table 7.5).

7.3.3.3. The effect of IBA and activated charcoal on the induction of adventitious roots

The effect of IBA (1 mgl^{-1}) and of a combination of IBA and activated charcoal (5.0 gl^{-1}) added to MS medium on the induction of adventitious roots was studied. Mechanical means (the use of either filter bridge or milcap plug) to support plantlets in the tubes were also tested.

Individual plantlets were either cultured on half-strength MS medium semi solid or liquid medium supported with either filter bridge or 'Milcap' plug or on MS semi-solid medium supplemented with IBA (1 mgl^{-1}) or a combination of IBA and activated charcoal

Table 7.5. The induction of adventitious roots from individual somatic embryos on medium supplemented with GA_3

State of medium	level of GA_3 (mgl^{-1})	% individual somatic embryos producing adventitious roots
semi-solid	-	6.7
	1.0	17.7
liquid	-	28.6
	1.0	14.3

Total χ^2= 2.4, with 3 degrees of freedom, giving P<0.05

Key

Number of replicates : 10

Basal medium : MS medium supplemented with 2% sucrose

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\mu Mm^{-2}s^{-1}$PAR

Culture procedure : 14 days on 4.0 mgl^{-1} 2,4-D containing medium, and a further 21 days on hormone-free MS medium before the somatic embryos were separated individually

(5.0 gl^{-1}) Table 7.6. shows the frequency of plantlets producing adventitious roots and the number of roots produced per plantlet.

Half-strength MS medium proved to be superior to that supplemented with either IBA alone or a combination of IBA and activated charcoal with respect to the number of roots produced per plantlet and the length of roots (Table 7.6). Plantlets growing on the latter medium, however, possessed darker green cotyledons and looked healthier than those on control medium.

Of the standard medium tested, liquid medium with filter bridge proved to be superior to semi solid medium and to that with milcap plug. By day 8, 70% of plantlets cultured in liquid medium supported with filter bridge rooted, while only 30% of those supported with 'Milcap' plug produced roots. Plate 7.1F. shows adventitious root formation from plantlets cultured in liquid medium supported with filter-paper bridge.

7.3.4. Hardening of somatic embryo-derived plantlets prior to transplantation

After being grown on half-strength MS medium for the induction of adventitious roots, plantlets were either transferred directly into pots containing Levington compost, or transferred into a growth container containing a 1:1 mixture of Levington compost and perlite, or into sterile plastic containers containing different mixture of growth media. In addition, the effect of the size of plantlets transferred directly to pot was investigated.

Table 7.6. The effect of IBA, activated charcoal and state of medium on the induction of adventitious roots of somatic embryo-derived plantlets

State of medium	level of ABA (mgl^{-1})	level of activated charcoal (gl^{-1})	% plantlets producing adventitious roots	number of roots/ plantlet	mean length of roots (cm)
semi-solid	-	-	50	4.2	9.1
semi-solid	1.0	-	60	4.8	1.0
semi-solid	1.0	5.0	70	2.4	5.0
liquid:					
*f.bridge	-	-	80	5.7	13.5
*M.plug	-	-	30	5.0	-

Key

Number of replicates : 10

Basal medium : half-strength MS medium

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\mu Mm^{-2}s^{-1}$PAR

Plate 7.1.

Development of somatic embryo-derived plantlets on hormone-free MS medium

A. Regeneration of clumps of somatic embryos of cassava cultivar CMC 76. x6.

B. Regeneration of clumps of somatic embryos of cassava cultivar TMS-83350. x4.5.

C. Regeneration of individual secondary somatic embryos. Note that each plantlet possesses a tap root. x 4.5.

D. Regeneration of plantlets from a fused somatic embryo. x 6

E. The induction of tap root on 'Milcap' plug saturated with half-strength MS medium supplemented with 2% sucrose. x4.5

F. The induction of adventitious roots on a filter bridge in half-strength MS medium before transfer in soil. x4.5.

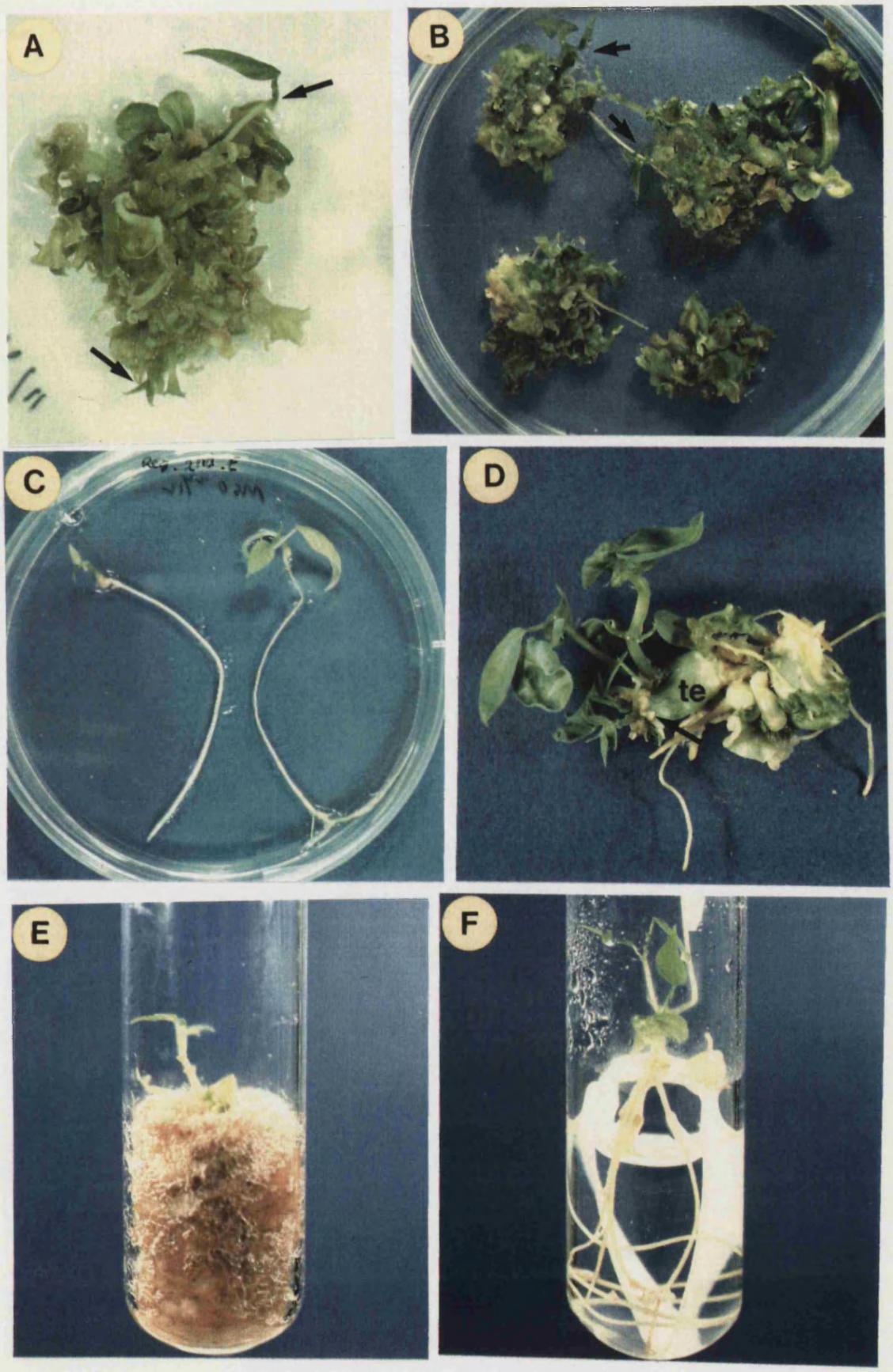
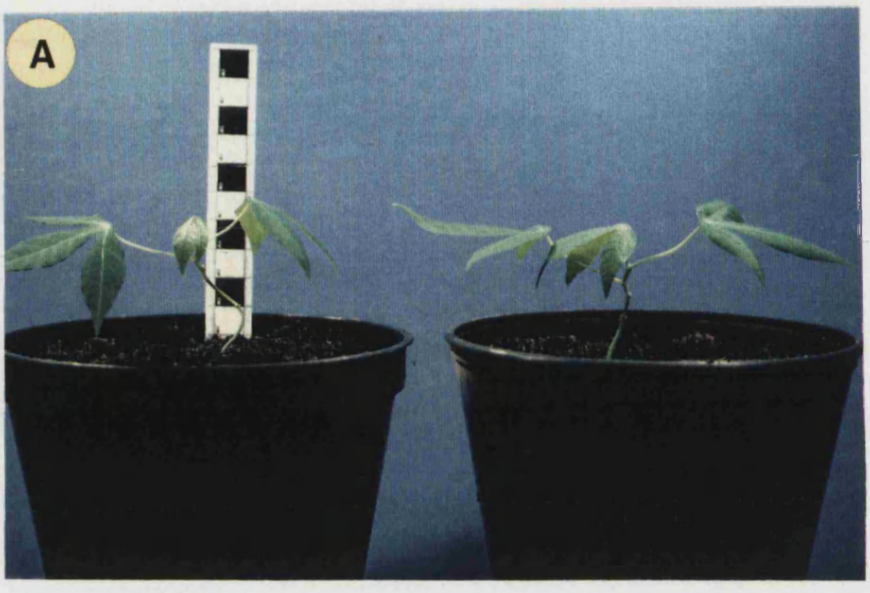

A
B
C
D
te
E
F

Plate 7.2.

Regeneration of plants derived from somatic embryos growing in pots containing Levington compost

A. Plants regenerated from somatic embryos after transplantation. x4.5

B. Mature plants regenerated from somatic embryos. x4.5.


A


CMC 76
CMC 76
PLANTS DERIVED FROM
SOMATIC EMBRYOS
B

7.3.4.1. The effect of the hardening procedure and growth compounds on the survival of plantlets in the green house

Three different sterile growth media (1:1 perlite and sand; 1:1 vermiculite and Levington compost, and Levington compost alone) placed in sterile plastic containers and saturated with half-strength MS medium were used to harden plantlets in a culture room under sterile conditions. Plantlets transferred directly to pots were either kept in the laboratory for one week or transferred directly to the greenhouse under a mist system. Those planted in mini propagators in the laboratory were hardened by gradually opening the lid over a period of one week.

Of the growth media tested for the hardening of plantlets under sterile conditions, the mixture of perlite and sand proved to be superior to the mixture of vermiculite and Levington compost or Levington compost alone (Table 7.7).

There was little difference between the perfomances of plantlets hardened under sterile conditions in the mixture of perlite and sand, and those placed directly in a growth container and those placed directly in pots in the laboratory; since all plantlets survived when they were transferred to the green house. Those hardened under sterile conditions, however, had a healthier appearance and they possessed thick, strong roots. Plates 7.2A. and 7.2B. show plants regenerated from somatic embryos after the transplantation to pots.

Table 7.7. The effect of hardening procedures and type of growth media on the survival following transplantation to the greenhouse

Hardening conditions	Growth media	% survival after 6 weeks
in Twyford boxes, in culture room (sterile conditions)	Perlite:sand = 1:1	100
	Vermiculite: L. compost = 1:1	70
	L. compost	70
in vivo:		
- in mini propagators, laboratory	Perlite: L. compost = 1:1	100
- in pots, laboratory	L. compost	100
- in pots, greenhouse mist system	L. compost	80

Total χ^2 = 9.8, with 5 degrees of freedom, $P<0.05$

Key: Number of replicates : 10

Liquid medium : half-strength MS medium

Temperature : $25\pm1^{o}C$, in a culture room

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

Table 7.8. Effect of the size of plantlets on the survival following transfer to the greenhouse

Size of plantlets (mm)	% survival after four weeks
20-40	70
40-60	100
60-80	50

Total χ^2 = 6.5*, with 2 degrees of freedom, giving $P>0.05$

Key : Number of replicates : 10

Growth media : perlite : Levington compost = 1:1

Hardening procedure : two weeks in the laboratory, room temperature

7.3.4.2. The effect of the size of plantlets on survival in the greenhouse

Plantlets with three different ranges of height (20-40, 40-60. and 60-80 mm) were compared in order to determine the optimum height for transplantation. The plantlets were directly transferred from test tubes in which they had been growing on half-strength MS medium to pots containing Levington compost and then kept in the laboratory at room temperature for two weeks. Table 7.8. shows the frequency of survival after the plantlets had been grown for a further four weeks in the greenhouse.

The best performance (100% survival) was obtained by transferring plantlets when their height was in a range of 40-60 mm. Larger plantlets rapidly lost the water so that the leaves wilted.

7.3.5. Chromosome counting of cassava somatic embryos and plantlets regenerated from somatic embryos

A number of pretreatment, fixation, hydrolysis and staining procedures, as listed in Table 7.9, were tested.

The most satisfactory procedure involved pretreatment with monobromonaphthalene for either 2 or 4 hours, fixed in Farmer's fixative (3 parts of 95% absolute alcohol and 1 part of acetic acid) irrespective of the duration tested, hydrolised in 1 N HCl for 10 minutes and stained with Feulgen solution for 20 minutes to 2 hours prior to staining with lacto propionic orcein.

Table 7.9. Summary of treatments for counting chromosomes of somatic embryos and of plantlets regenerated from somatic embryos of cassava

Type of tissue	Pretreatment type/duration	Fixation type/duration	Hydrolysis type/duration	Staining type/duration
somatic embryos and root tips	3mM 8-hydroxy quinoline or monobromo-naphthalene for 2 or 4 h at 22°C	Carnoy's or Farmer's fixative for 2, 12, or 24 h at 22°C	1 N HCl or 1% pectinase+ 1% cellulase for 10, 20 or 30 min at 60°C	aceto carmine (1% or 2%),or Feulgen and 60% lacto-propionic orcein for 10, 20 min or 2h at 22°C

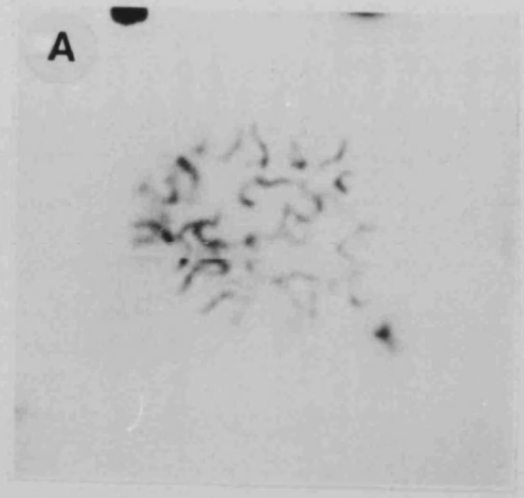
The number of chromosomes counted of both somatic embryos and root tips was 2n=36. There was apparently no variations could be observed from five counts made. Plate 7.3A,B. show metaphase chromosomes from somatic embryo and plantlet.

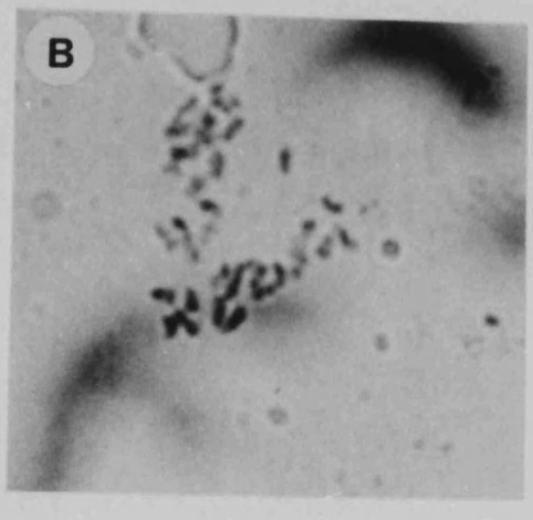
Plate 7.3.

Metaphase chromosomes of somatic embryos and of root tips of plants derived from somatic embryos of cassava.

A. Metaphase chromosomes from somatic embryos. x3094

B. Metaphase chromosomes from root tips. x3094.


A


B

7.4. DISCUSSION

The production of plantlets possessing a normal shoot and roots was affected by the size of somatic embryos when they were cultured on regeneration medium (Section 7.3.1). The effect of the size of somatic embryos on successful plantlet production has also been demonstrated by Trolinder and Goodin (1988) in work with cotton. They found that the highest survival (30%) could be obtained if the size of somatic embryos was in the range 5-10 mm when they were cultured in rooting medium before they were transferred to pots. In the case of cassava, somatic embryos larger than 3 mm at the time they were separated individually and cultured on regeneration medium had a lower rate of survival which could presumably be attributed to the failure of shoot development. Larger somatic embryos, particularly those larger than 7 mm have possessed large dark green cotyledons due to precocious germination which apparently caused abnormal development (Section 7.3.1). Kerns *et al* (1986) and Ammirato (1987) also stated that malformed or aborted plants are a result of precocious germination. On the other hand, the poor performance of plantlets regenerated from somatic embryos smaller than 1 mm indicated that they were not capable of independent existence after separation from the clumps in which they had developed; at least, not on this particular regeneration medium.

Size would also seem to play an important role in the success of transplantation from *in vitro* conditions to the greenhouse. The rate of survival was affected by the height of normal plantlets possessing both shoot and root (Section 7.3.4.2). Maene and Debergh (1983) showed that 25-60 mm was the optimum size of plantlets of *Cordyline*

terminalis for transfer to the greenhouse. They suggested that plantlets larger than 60 mm have too great a leaf surface, and that some rotting and losses occur as a result of a non-functional root system being developed *in vitro*. Conversely, Tisserat (1982) demonstrated that plantlets regenerated from date palm somatic embryos larger than 100 mm gave the highest survival rates because they had several expanded leaves and a well-developed root system composed of adventitious roots. This contradictory evidence suggest that different species requires different optimum size for transplantation.

The success of transplantation would also seem to be affected by the development of the root system *in vitro*. The induction of adventitious root *in vitro* using GA_3 would be beneficial if GA_3 was added to semi solid medium (Section 7.3.3.2). In liquid medium the addition of GA_3 gave rise to the reduction of the frequency of rooting. This contradictory effect of GA_3 which was also noted by other investigators is not really understood. Cleland (1969) considered that GA_3 is not a major factor in initiation and subsequent root development and that in many cases its application has no effect. Mitsuhashi *et al.*(1969), on the other hand, demonstrated that GA_3 inhibited rooting of cuttings from various species, while Anand *et al.*(1972) found that GA_3 enhanced rooting of *Ipomea fistulasa* cuttings. This contradictory observations concerning the use of GA_3 may result from differences in response to exogenous gibberellins in the different plant species. It would also seem that the effect of GA_3 is dependent upon other hormones as it was reported by Smith and Thorpe (1975) that in the presence of IBA, GA_3 had no effect on root initiation.

Ammirato (1977) demonstrated that GA_3 in combination with ZEA and ABA promoted more normal development of caraway somatic embryos. Different effects of

GA_3 would also be caused by the difference in time of application. Gibberellic acid inhibited meristemoid formation if it was applied over the first four days, but it stimulated rooting of hypocotyls of *Pinus radiata* if it was given over days 4 to 6 days (Smith and Thorpe, 1975b). Histologically, it was observed that the application of GA_3 over this period enhanced the formation and/or development of the loci about which meristemoids differentiate (Smith and Thorpe, 1975a). This time dependence was in agreement with results in Sections 7.3.2 and 7.3.3.2 and showed that GA_3 added to semi- solid MS medium used for growing clumps of somatic embryos (three weeks old) accelerated the growth of somatic embryos but inhibited root initiation, while GA_3 added to the medium for culturing individual somatic embryos (six weeks old) initiated roots. The maturation of somatic embryos of *Citrus sinensis* (Kochba *et al.*,1979), of *Panicum maximum* (Lu and Vasil, 1981), of *Zea mays* (Lu *et al.*, 1982) was also fostered by the addition of GA_3. Kavatekhar *et al.*(1978); Rajasekaran and Mullins (1979) reported that gibberellic acid was capable of breaking somatic embryo dormancy in *Eschescholzia californica* and other plant species, thus to substituting for chilling treatment.

The frequency of normal plantlet production was also affected by ABA (Section 7.3.2). The results were in agreement with those of Nitsch and Nitsch (1969); Kamada and Harada (1981); Ammirato (1983) showing that somatic embryos of tobacco and carrot, respectively, grown with ABA tend to have a high frequency of embryos with two cotyledons. This normalizing effect of ABA has also been observed with *Pennisetum americanum* (Vasil and Vasil, 1981).

It was also shown that the induction of adventitious roots did not seem to be affected by IBA alone (1.0 mgl^{-1}) or by a combination of IBA and activated charcoal (Section 7.3.3.3). On medium with IBA alone, the induced root were thick and their growth was very slow. Jones and Hatfield (1976) and James (1979) pointed out that a concentration

of 5 x 10^{-6} M was too high if IBA alone were to be used for rooting. Based on this point of view, the concentration of IBA used was probably too high for rooting plantlets derived from cassava somatic embryos.

It seems that different plant species respond differently towards rooting compounds. Roots of *Pinus radiata* (Smith and Thorpe, 1975), of *Picea abies* (von Arnold, 1982), and of *Cymbopogon martinii* (Barual and Bordoi, 1989) were initiated with IBA at 2.0 mgl^{-1}, while IAA (0.1-2,0 mgl^{-1}) was used to initiate roots of somatic embryos of cotton (Trolinder and Goodin, 1988), and of *Petunia hybrida* (Colijn *et al.*, 1979). NAA (0.1-2.0 mgl^{-1}) was shown to be beneficial for rooting somatic embryos of date palm (Tisserat, 1982), of *Petunia hybrida* (Colijn *et al.*, 1979) of *Cymbopogon martinii* (Barual and Bordoi, 1989) and *Sorghum almum* (George and Eapen, 1988). On the other hand, Murashige and Skoog (1962) standard medium devoid of hormones or rooting compounds was used for rooting shoots regenerated from root meristems of *Petunia hybrida* and for those regenerated from protoplasts of *Capsicum annum* (Diaz *et al.*, 1988).

The response of cassava plantlets towards activated charcoal seems to be contradictory to that of other plant species. The results (Section 7.3.3.3) showed that plantlets grown in medium supplemented with activated charcoal in combination with IBA only produced 2.4 roots in average, whereas Fridborg and Eriksson (1975) could induce abundant amounts of root formation of *Allium cepa* with 1-4% activated charcoal. Plantlets grown in this medium, however, looked healthier and they possessed thick shoots and darker green leaves (Section 7.3.3.3). Apparently, activated charcoal can also play an important role in the normal development of somatic embryos. Fridborg *et al.*(1978); Chee and Tricoli (1988) believed that medium supplemented with activated charcoal reduced the the number of abnormal somatic embryos; the somatic embryos

obtained from suspension culture cells, without prewashing with charcoal, developed into plantlets with abnormal primary leaves.

The fact that direct transfer to pots containing Levington compost in the laboratory gave the same result as hardening plantlets in a culture in a mixture of sterile perlite and sand (1:1) suggests that cassava plantlets derived from somatic embryos do not require a complicated hardening procedure. This result accords with that of Yeh and Chang (1987) in that somatic embryos of *Sinocalamus latiflora* can be succesfully transplanted in soil without hardening.

The results (Section 7.3.5) demonstrated that as far as chromosome number was concerned, there was no evidence of genetic variability among the plants regenerated from somatic embryos of cassava. This was despite the fact that the embryogenic cultures had been maintained up to 6 months on medium containing 4.0 mgl^{-1} 2,4-D. This was not in agreement with the evidence from other work that 2,4-D induced both mitotic and meitotic irregularities in a number of plant species (Unrau and Larter, 1952). Anaphase anomalies in carrot cell suspension cultures was reported induced by 2,4-D (Bayliss, 1973). Singh and Harvey (1975), however, demonstrated a positive effect of 2,4-D on cell suspension of *Hapopaplus gracilis* and of *Vicia hajastana*, that the higher the concentration of 2,4-D, the lower the frequency of abnormal anaphase configurations.

CHAPTER 8

CRYOPRESERVATION OF CASSAVA SOMATIC EMBRYOS AND EMBRYOGENIC TISSUES

8.1. INTRODUCTION

The gradual loss of morphogenetic competence, frequently encountered in continuosly growing cultures, is a problem that can limit the exploitation of embryogenesis system. Until there is a better understanding of the factors causing the loss of competence, it is important to develop methods which can be used to store the embryogenic cultures in a non-growing state. It is very likely that cryopreservation techniques will provide the only practical solution to this problem and, indeed, it is possible that the cryopreservation of somatic embryos could in addition provide a valid method of germplasm storage in certain species.

8.1.1. The History of Cryopreservation

The growth rate of cultures is an important factor in long-term storage. Slow growth, induced by those of growth inhibitors, e.g., ABA, osmotic agents e.g., mannitol, or cool treatments (Ammirato, 1983; Westcott, 1981a,b), extends the subculture interval, and is therefore of value for long term genetic conservation. However, conditions which completely inhibit growth without causing damage would be of even greater value. Storage at -80^{o}C may be satisfactory in the short to medium term since it would maintain intracellular freezing; however, it is known that ice is mobile, in the sense of migration of water molecules from one ice-crystal to another, down to approximately -120^{o}C and this could result in ice crystal growth and cell damage. For this reason, cryopreservation involving storage at the temperature of liquid nitrogen (-196^{o}C) is apparently the only method that can be recommended for long term conservation of vegetative germplasm under non-growing conditions (Withers, 1978; Henshaw, 1982; Bajaj, 1985).

Cryopreservation, one of the most promising approaches now being pursued to prevent progressive changes in the genome which is an important factor to maintain genetic stability of long-term preservation of plant material (Kartha, 1984). It is argued (Tisserat *et.al.*, 1981) that storage at -196^{o} C suppresses cell division and DNA synthesis for an indefinite period of time, and that at this temperature, as a result of the reduction of most physical and chemical processes to negligible levels, long term stability is assured except for the minor risk of damage resulting from background radiation (Whittingham *et.al.*, 1977).

Cryopreservation techniques were first used successfully with plant cultures when Quatrano (1968) froze the cells of flax (*Linum usitatissimum*) to a temperature of -50^{o}C and maintained viability at a level of 14%. This finding was an important contribution for the development of cryopreservation techniques, although it is apparent from both theoretical and practical consideration that, -50^{o}C is not a sufficiently low enough temperature for stable long-term storage (Meryman and Williams, 1982). Subsequently, regeneration of frozen cultured cells, tissues or organs of higher plants has been reported in, for example, carrot (Nag and Street, 1973; Dougall and Wetherell, 1974; Withers, 1979), rice and sugarcane (Finkle and Ulrich, 1982); date palm (Tisserat *et.al.*, 1981); potato meristems (Grout and Henshaw, 1978; Bajaj, 1981; Towill, 1983; Manzhulin *et.al.*, 1984; Bajaj, 1985; Henshaw *et.al.*, 1985), pea meristems (Kartha *et.al.*, 1979), and strawberry meristems (Sakai *et.al.*, 1978), and cassava meristems (Kartha *et.al.*, 1985).

8.1.2. Basic principles of cryopreservation

Cryopreservation procedures involve a number of stages : culture before freezing, cryoprotectant application, freezing, storage, thawing and culture on regeneration

medium. Factors influencing any one of these stages can affect the success of the cryopreservation procedure, together with other factors including the size, age and genotype of the explants or cultures subjected to the procedure. At present, it would seem that the optimal combination of conditions for a particular genotype can only be established empirically.

8.1.2.1. Culture before freezing

Depending on the genotypes used, work with potato shoot tips showed that there was a definite requirement for a short culture period before freezing. One to four days were considered as the optimum length of time to allow the shoot tips to recover after the excision without permitting so much growth (Stamp and Henshaw, 1982). Manzhulin *et.al.* (1984) showed that explants incubated on medium containing 5% (v/v) DMSO for a period of 24 hours gave the highest recovery rate. It has also been suggested that a higher recovery rate may possibly be achieved by culture not only in the presence of cryoprotectant but also in media of enhanced osmotic potential (Nag and Street, 1975).

Orr *et.al.*, (1986) reported that the use of ABA to induce freezing tolerance in *Brasicca napus* has the advantage of decreasing a hardening period from four weeks to one week. A higher level of freezing tolerance was induced when embryogenic cells were cultured for 7-8 days in 5 X 10^{-5}M ABAand 13% sucrose at 25^{o}C (60-70% survival at -20^{o}C) than when cells were cultured in the same medium for 4 weeks at 2°C (40% survival at -20^{o}C). Light microscopic observations showed that vacuoles were notably smaller or absent from most of the hardened cells. Ultrastructurally, the majority of hardened cells were characterized by a proliferation of numerous small cytoplasmic vesicles. It has also been reported that the presence of 13% sucrose alone induced a

level of hardening similar to that produced by 6.5% sucrose plus ABA (25% survival at -20°C) whereas 6.5% sucrose alone was ineffective.

There are other reports of the phenomenon of cold hardening being exploited to permit successful freeze preservation in otherwise sensitive specimens. Marked improvements in the survival of frozen and thawed callus of *Populus euramericana* (Sakai and Sugawara, 1973) and meristems of *Dianthus caryophyllus* (Seibert and Wetherbee, 1977) have been achieved by the application of cold pretreatments. The physiological basis for survival may relate to a changed level of intracellular solutes and altered cell membrane composition. Steponkus *et.al.*, (1983) concluded that the lower incidence of intracellular ice formation in acclimated protoplasts is the result of a lower nucleation temperature.

8.1.2.2. Cryoprotectant application

The application of cryopotectant compounds is essential to the survival of frozen explants. A cryoprotectant with a larger molar volume, such as DMSO or glycerol , can protect against cell volume loss over and above its pure colligative effect. On the other hand , cryoprotectants, particularly DMSO, are thought to be potentially deleterious upon long exposure, and for this reason their application has frequently been carried out at a reduced temperature (c. 0-4°C) and for a brief period of 1-2 hours before freezing.

Meryman and Williams (1977) pointed out that the cryoprotectant must reach the interior of the cell, otherwise it will cause osmotic dehydration, and that it must be non toxic in concentrations sufficiently high to produce a useful amount of freezing-point depression. However, the presence of cryoprotectant in the external medium during

initial cooling may also be important, as indicated by the rapid loss of the protectant effect by suspension in the protectant-free medium.

DMSO has proved to be effective in preventing freezing injury in a number of plant cell cultures. It fulfils all the criteria of an efficient cryoprotectant : it has a low molecular weight, is easily miscible with water, can be easily washed away, and it permeates rapidly into the cells (Bajaj, 1976). Concentrations in the range of 5 to 10% (v/v) seem to be optimal for most cells and organs (Quatrano, 1968; Lalta, 1971; Nag and Street, 1973; Dougall and Wetherell, 1974; Bajaj and Reinert, 1975; Towill, 1983; Withers, 1984). A combination of two cryoprotectants provided a better result than a single cryoprotectant. Towill (1983), for example, reported that the combination of 5% DMSO and 5% glycerol gave 46% survival at -40^{o}C in contrast to 0% survival for either 5% DMSO or 5% glycerol used alone.

8.1.2.3. Freezing

Freezing techniques which have been used successfully with plant, cell and organ cultures can be catagorised into : slow-freezing, rapid freezing, and two-stage freezing involving initial slow-freezing to approximately -30^{o}C before transfer to liquid nitrogen.

8.1.2.3.1. Slow freezing

Slow freezing (ca. 0.1-1.0^{o}Cmin^{-1}) leads to extracellular ice formation which, in turn, causes the osmotic efflux of intracellular water. A carefully selected slow rate of cooling is required to permit the extraction of sufficient water without causing damage

through increased solute concentration before intracellular freezing ensues. Slow freezing has resulted in the successful cryopreservation of a number of plant species such as chickpea and groundnut (Bajaj, 1979; Kartha, 1984).

8.1.2.3.2. Rapid freezing

Rapid-freezing methods in which cooling rates of the order of several hundred degrees per minute are obtained by freezing the sample directly in liquid nitrogen. It is believed that during rapid freezing, the viability of cells is maintained by the prevention of the heterogenous growth of intracellular ice crystals as the tissue passes rapidly through the temperature zone in which lethal ice crystal growth occurs (Luyet's mechanism, quoted by Seibert and Wetherbee, 1977). Kartha, 1984 argues that although rapid freezing methods may be applicable to a very limited number of plant species, a generalization concerning the universality of their application to all species cannot be made.

8.1.2.3.3. Two-stage cooling

This method seems to combine the advantages of the other two methods, since partial dehydration is achieved before homogenous ice-crystal formation occurs within the cells. The successful use of this method with high recovery rates has been reported in potato meristems (Towill, 1981; Henshaw *et.al.*, 1985) and date palm (Engelmann *et.al.*, 1985). According to Kartha (1984), viability is influenced by the terminal freezing temperature for the first stage before samples are transferred directly to liquid nitrogen. It is considered that -30°C to -40°C is a safe range for meristems of most species.

8.1.2.4. Thawing

Thawing rates are also critical in determining the recovery rate of frozen explants, with the choice between rapid or slow thawing procedures being dependent upon the rate of cooling. According to Henshaw (1982) rapidly-cooled specimens must also be thawed rapidly because of the risk of ice-recrystallisation, whereas optimal thawing rates for slowly-cooled specimens probably depend on the degree of dehydration that they have undergone and the amount of intra-cellular ice that has been formed. Slow thawing has advantages for relatively large specimens which are brittle when frozen, since it reduces the risk of physical damage. Withers (1979) stated that the thawing of frozen plantlets by placing directly on to semi solid medium (the washing stage was omitted) produced a dramatic increase in both the rate and the frequency of recovery growth.

It has also been suggested that the damaging effects of thawing may be reduced by washing with medium which had been warmed at 25^{o}C (Finkle and Ulrich, 1982), or by washing with medium supplemented with the same additives as those used in the medium for growth before freezing (Maddox *et.al.*, 1983).

8.1.2.6. Culture regeneration medium

The composition of the recovery medium and the culture conditions are further critical factors that can influence the recovery of frozen specimens. Work with potato meristems (Towill, 1981) indicated that the use of a callus inducing recovery medium

might have been an important factor since it would have led to the rescue of partially damaged meristems which would have been incapable of organized growth.

8.1.3. Cryopreservation of Somatic Embryos

Whilst it is well-known that zygotic embryos in 'orthodox' seeds with low water content are relatively amerable to cryopreservation techniques, work on the cryopreservation of somatic embryos has been confined to a restricted number of species. Bajaj (1976) successfully cryopreserved young globular embryos from carrot cell suspension cultures. Normal-looking plants producing healthy roots were grown from cultures stored up to 8 months in liquid nitrogen (-196^{o}C), but the survival of embryos decreased with advances in their stage of development. Using a method in which the material was frozen in a "dry" state rather than immersed in medium, Withers (1979) obtained improved survival of older carrot embryos and young plantlets.

In addition to carrot somatic embryos, successful cryopreservation of citrus somatic embryos and oil palm somatic embryos has also been reported. Marin and Duran-vila (1988) obtained high frequency of surviving plants derived from frozen citrus somatic embryos which had been slow-cooled at a rate of 0.5^{o}Cmin^{-1} down to -42^{o}C prior to storage in liquid nitrogen. At two-stage cooling method was also used to cryopreserve the oil palm somatic embryos which had been grown in a medium containing a high sucrose concentration (0.3M) for 7 days prior to freezing (Engelmann *et.al.*, 1985).

The difficulties encountered in cryopreserving somatic embryos are mainly due to their size and water content, with complete organs and large-sized embryos being

particularly vulnerable to cryodamage (Bajaj, 1985). Thus, the water content of these embryos needs to be controlled to bring it to a level at which intracellular freezing is minimal. The very young and immature embryos perhaps do not need any special manipulation, but the fully differentiated, more mature embryos have to be subjected to partial dehydration for successful preservation.

DMSO at a concentration of 5% to 10% (v/v) is commonly employed as a cryoprotectant with somatic embryos and a mixture of DMSO, sucrose and glycerol at low concentrations (5% v/v of each) has given encouraging results (Withers, 1979; Bajaj, 1985). The survival of somatic embryos, however, is mainly determined by their stage of development.

8.1.4. Cryopreservation in Cassava

Cryopreservation in cassava has only been carried out with seeds and meristems. Mumford and Grout (1978) reported that 23% of frozen cassava seeds immersed directly in liquid nitrogen were able to germinate. Higher germination percentages of up to 80% could be obtained by freezing the seeds in the vapour above liquid nitrogen. Kartha *et.al.* (1982) cryopreserved cassava meristems treated with a 15% (v/v) DMSO and 3% (w/v) sucrose solution for 15 minutes. They were frozen over an 18 μm aluminium foil in 2-3 μl droplets of the cryoprotectants solutions in plastic Petri dishes at a cooling rate of $0.5^{o}Cmin^{-1}$ to various sub-zero temperatures (-20, -25, -30 and -45^{oC}) and stored in liquid nitrogen ($-196^{o}C$). The thawed meristems exhibited various morphogenetic responses such as differentiation of callus and leaves and whole plantlets when they were cultured on regeneration medium (MS + 0.5 M BA + 1 M NAA + 0.1 M GA_3, 0.8% Bacto Difco Agar) at $26^{o}C$, with a 16 hour photoperiods at 4000 lx. Plants obtained from

excised cassava meristems cryopreserved for up to 4 years were obtained by Bajaj (1985). The percentage of survival was 34.4% which was about the same as that of meristems cryopreserved for 3 months only.

8.1.5. Approach Used in Cryopreservation of Cassava Somatic Embryos

The two-stage cooling procedure that was used was based on the technique developed for potato meristems by Henshaw *et.al.*(1985). The samples were slow-cooled at a rate of $0.3^{o}C\ min^{-1}$ prior to the immersion in liquid nitrogen ($-196^{o}C$).

It was thought that a cooling rate of $0.3^{o}C\ min^{-1}$ might be suitable for somatic embryos of cassava since it was similar to the cooling rate of $0.5^{o}C\ min^{-1}$ used successfully with other plants such as oil palm (Engelmann and Dereuddre, 1988) and citrus (Marin and Duran-villa, 1988).

The decision to culture embryogenic tissue or somatic embryos of cassava on a high sucrose concentration medium for several days before freezing was also based on the protocols for somatic embryos of oil palm (Engelmann and Dereuddre, 1988) and of citrus (Marin and Duran-villa, 1988) which were cultured on medium supplemented with 5% and 25% sucrose respectively.

Preliminary investigations were carried out to determine the smallest size of cassava somatic embryos which were capable of regenerating, the optimum duration of culture before freezing, and the optimum concentration of cryoprotectants and of sucrose.

8.2. MATERIALS AND METHODS

8.2.1. Source of explants

The types of explant used for cryopreservation included both embryogenic tissues and individual somatic embryos derived from primary somatic embryos which were induced from young leaf lobes of cassava cultivar CMC 76. The embryogenic tissues used were tissues with nodular-like structures, while the individual somatic embryos used were those "torpedo-shape" in structure. The approximate size of both tissues were 1.5-2.0 mm.

8.2.2. Culture Before Freezing

Batches of of six embryogenic explants in the size range of 1.5-2.0 mm placed on 8 x 27 mm lens tissue aluminium foil carriers (Figure 8.1) were incubated for four days prior to freezing on semi-solid media containing appropriate concentration of 2,4-D and sucrose.

8.2.3. Application of Cryoprotectants

Unless otherwise stated, tissues were treated with DMSO at 10% (v/v) in MS medium for 2 hours before freezing. Explants in lens tissue aluminium foil containers were transferred to empty Petri dishes, two drops of cryoprotectant solution were dropped using Pasteur pippete on the carriers. The top layer of carriers was then

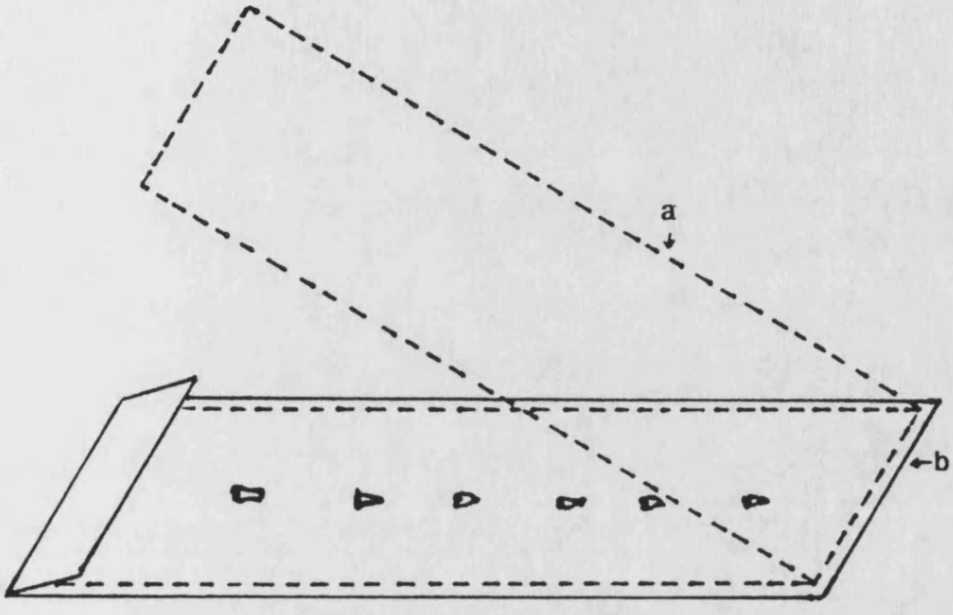


Fig. 8.1. Lens tissue-aluminium foil carrier

Key :

a. Lens tissue
b. Aluminium foil

Plate 8.1.

Freezing apparatus

a. Temperature programmer

b. methanol bath

c. cryocool

d. thermostatiscally controlled cooling coil

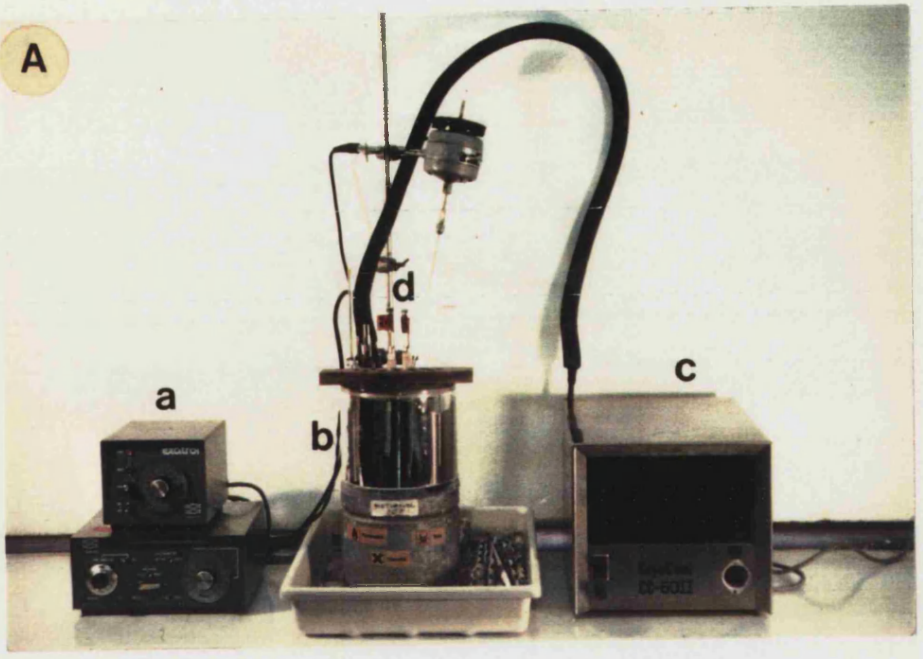
e. temperature probe

f. cane

g. thermometer

h. stirrer

A



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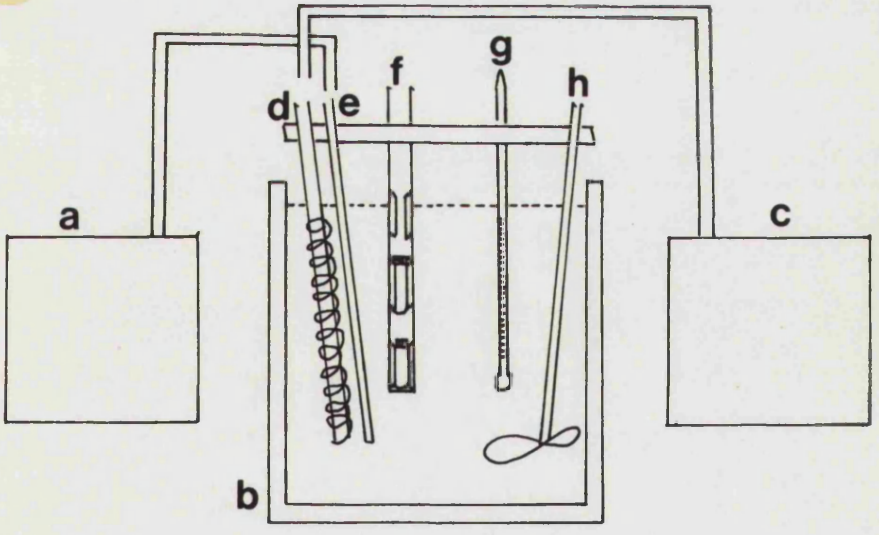


layered on top of explants. All bubbles were removed from the carriers by using forceps. After two hours, the carriers were then placed in 1.8 ml cryotubes (Nunc.) to be frozen.

8.2.4. Freezing procedures

In most experiments, explants were subjected to a two-step freezing procedure using the apparatus illustrated in Plate 8.1.

During the first stage, cryotubes containing explants were fixed on "canes" which were loaded into a methanol bath running at -5^{o}C. After allowing it to stabilise for 10 minutes, ice formation in the cryoprotectant solution was induced by touching the base of the cryotubes with liquid nitrogen-cooled forceps. They were then allowed to stabilise for a further 10 minutes at -5^{o}C (at a cooling rate of 12^{o}C min^{-1}) before they were cooled to -30^{o}C at a cooling rate of 0.3^{o}C min^{-1}.

For the second stage, after the temperature reached -30^{o}C, the cryotubes were removed from the methanol bath and the carriers were quickly taken out of the tubes prior to immersion in liquid nitrogen (-196^{o}C) for 15 minutes.

8.2.5. The Thawing and Rinsing Procedure

The carriers were removed from the liquid nitrogen and plunged into 5 ml of liquid MS culture medium containing the same composition as that for culturing explants before freezing at 22^{o}C. Although thawing occured in a few seconds, the explants were

left to rinse for one hour in the same liquid medium before they were plated out onto semi solid regeneration medium.

8.2.6. Culture After Freezing

Thawed explants were cultured at $25\pm1^{o}C$ under 16 hours photoperiod (40 $Mm^{-1}s^{-1}$PAR) in 5 ml Petri dishes containing 10 ml medium of the same composition as that used for culturing tissues before freezing.

The sucrose concentration was reduced by 20 gl^{-1} at 3 day intervals to a final concentration of 2% (w/v).

8.2.11. Survival Assessment

Two weeks after freezing, survival was assessed on the basis of embryo growth, callus production and new somatic embryo production.

8.3. RESULTS

8.3.1. Preliminary Investigation

Because of the importance of the physiological state and the size of explants that were subjected to cryopreservation procedures, preliminary experiments were designed to establish the type of explant that might be used in the subsequent experiments. The intention was to identify small embryogenic explants which responded vigorously to the standard culture conditions that were employed in association with the cryopreservation procedures. Ideally, different explants should be tested along with each of the procedures but this compromise was adopted in order to keep the size of the experiments within manageable proportion.

8.3.1.1. The effect of the type of embryogenic explants and 2,4-D concentration on the survival and growth of somatic embryos

Since it was not certain that intact somatic embryos of a sufficiently small size for cryopreservation purposes could be reliably subcultured, the effect of 2,4-D on alternative embryogenic explants was tested. These explants included pieces of embryogenic tissues and parts of somatic embryos, cut approximately in the region of the hypocotyl which is not readily distinguishable.

Low concentrations of 2,4-D (0.01 and 0.05 mgl^{-1}) were used with the media and the explants were cultured in the light (40 $Mm^{-1}s^{-1}$PAR) or in the dark.

Table 8.1. The effect of part of somatic embryos and the light on the survival rate

Incubation condition	part of embryos	% somatic embryos remained the same	% somatic embryos continuing growth	% somatic embryos producing new embryos
light	top	40	40	20
	bottom	70	0	0
dark	top	30	40	0
	bottom	70	0	0

Table 8.2. The effect of the type of explant and the concentration of 2,4-D on the survival and growth of somatic embryos

Type of explant	Level of 2,4-D (mgl^{-1})	Length (mm)	% somatic embryos remained the same	% somatic embryos continuing growth	% somatic embryos producing new embryos
embryogenic tissues	0.01	0.5-1.0	10	80	10
	0.05	0.5-1.0	30	40	0
somatic embryos:					
- top	0.01	0.5	30	70	0
	0.05	0.5	40	60	20
- bottom	0.01	0.5	80	0	0
	0.05	0.5	80	0	0
- whole part	0.01	0.5	40	60	10
	0.05	0.5	30	70	20

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\,\mu Mm^{-2}s^{-1}$PAR

No new embryos were produced by embryogenic tissues cultured on medium supplemented with 0.05 mgl^{-1} 2,4-D. With the lower level of 2,4-D (0.01 mgl^{-1}), 10% of those tissues were able to undergo further embryogenesis (Table 8.2).

Unlike embryogenic tissues, 20% of somatic embryos (either the top part or the whole part) could produce new embryos on medium supplemented with 0.05 mgl^{-1} 2,4-D.

There was no difference between the top parts of embryos cultured in the light and in the dark in terms of the growth of somatic embryos (Table 8.1). Culturing them in the light, however, was more beneficial with regard to the production of secondary embryos (up to 20%), while those cultured in the dark were not able to undergo embryogenesis.

8.3.1.2. The effect of the size of somatic embryos and the concentration of 2,4D on the survival and growth of somatic embryos

Successful cryopreservation of cassava somatic embryos is most likely to be achieved with the smallest explants that will produce secondary somatic embryos under normal conditions. The growth and development of somatic embryos of four different ranges of sizes (0.5-1.0, 1.0-1.5, 1.5-2.0, 2.0-2.5 mm in length) was, therefore, investigated on a range of media containing either 0.01, 0.05, 0.10, 0.50, 1.00, or 5.00 mgl^{-1} 2,4-D.

The highest frequency (90%) of surviving embryos was obtained by culturing embryos either 1.5-2.0 mm or 2.0-2.5 mm in length on medium supplemented with 0.05 mgl^{-1} 2,4D (Table 8.3). The 1.5-2.0 mm somatic embryos, however, proved to be the

Table 8.3. The effect of the size of somatic embryos and concentration of 2,4-D on the survival and growth of somatic embryos

Medium (level of 2.4D (mgl^{-1})	Length of embryos (mm)	%embryos remain the same	%embryos producing secondary embryos	%embryos continuing growth	% total survival
0.01	0.5-1.0	40	10	40	40
	1.0-1.5	20	20	40	50
	1.5-2.0	30	30	50	60
	2.0-2.5	20	20	50	60
0.05	0.5-1.0	30	20	40	50
	1.0-1.5	10	30	60	70
	1.5-2.0	30	50	40	90
	2.0-2.5	20	40	50	90
0.10	0.5-1.0	50	10	30	30
	1.0-1.5	40	20	40	50
	1.5-2.0	20	20	40	50
	2.0-2.5	20	30	50	60
0.50	0.5-1.0	70	0	20	20
	1.0-1.5	50	10	30	30
	1.5-2.0	30	20	40	50
	2.0-2.5	30	20	40	60
1.00	0.5-1.0	100	0	0	0
	1.0-1.5	70	0	20	20
	1.5-2.0	50	10	20	20
	2.0-2.5	40	30	30	40
5.00	0.5-1.0	100	0	0	0
	1.0-1.5	100	0	0	0
	1.5-2.0	90	10	0	10
	2.0-2.5	80	20	0	20

Overall level $\chi^2_{(survival)} = 48.1^{**}$, with 5 degrees of freedom, giving $P>0.01$

Key :

Number of replicates : 10

Basal medium : MS supplemented with 2% (w/v) sucrose

Temperature : $25\pm1^{0}C$

Light conditions : 16 hours photoperiod,

$30\mu Mm^{-2}s^{-1}$PAR

Plate 8.2.

Embryogenic tissues and somatic embryos before and after freezing

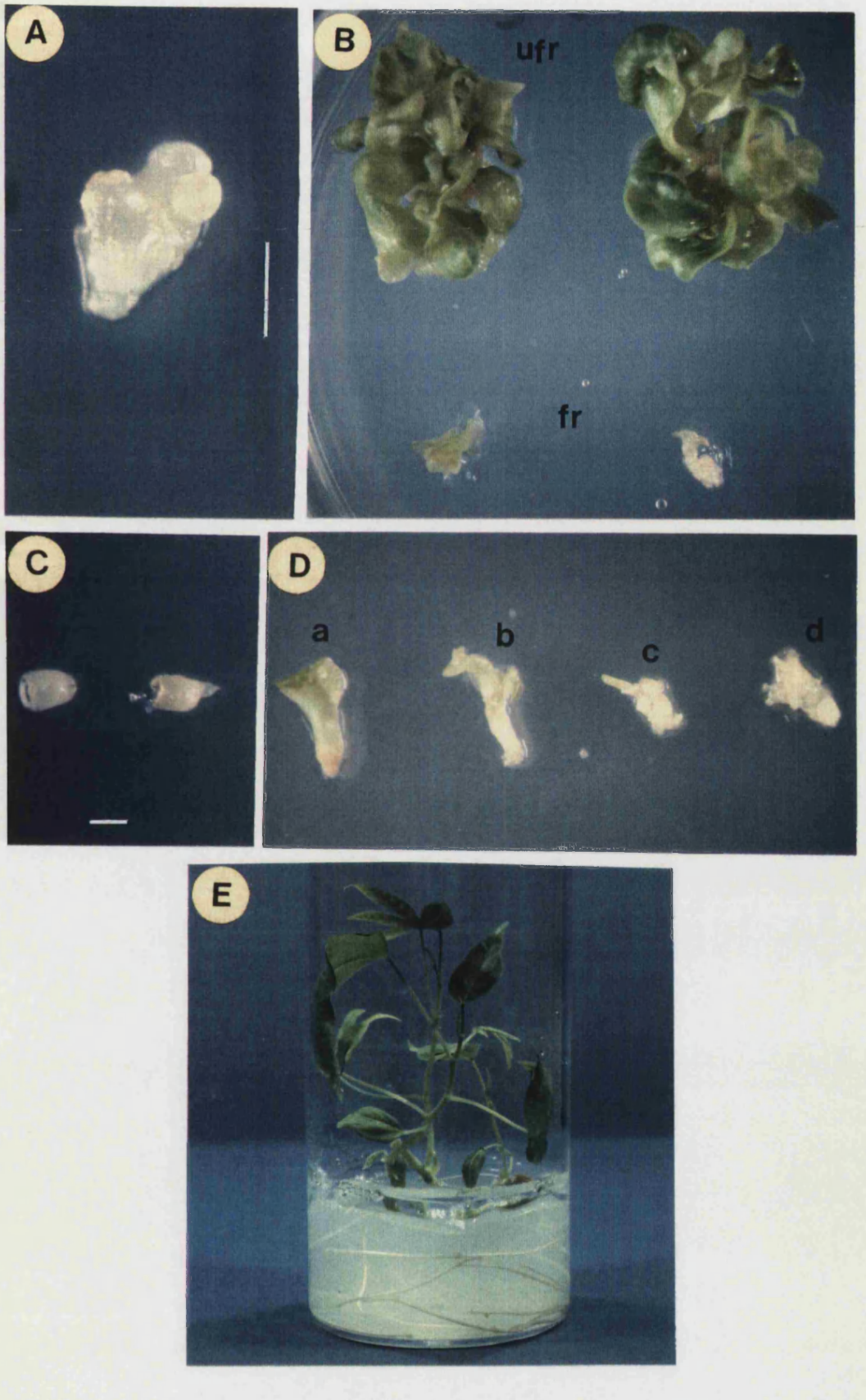
A. Type of embryogenic tissue used in cryopreservation experiment. Scale unit = 1 mm

B. Regeneration of frozen embryogenic tissues subjected to two stage cooling procedure. x16.2

C. Type of somatic embryos used in cryopreservation experiment. Scale unit = 1 mm

D. Types of regeneration of frozen somatic embryos : (a) frozen somatic embryos showing continued growth, (b) frozen somatic embryos showing continued growth and production of secondary embryos, (c) frozen somatic embryos producing secondary embryos. x16.2.

E. Plantlet regenerated from frozen somatic embryos. x4.5


A
B
ufr
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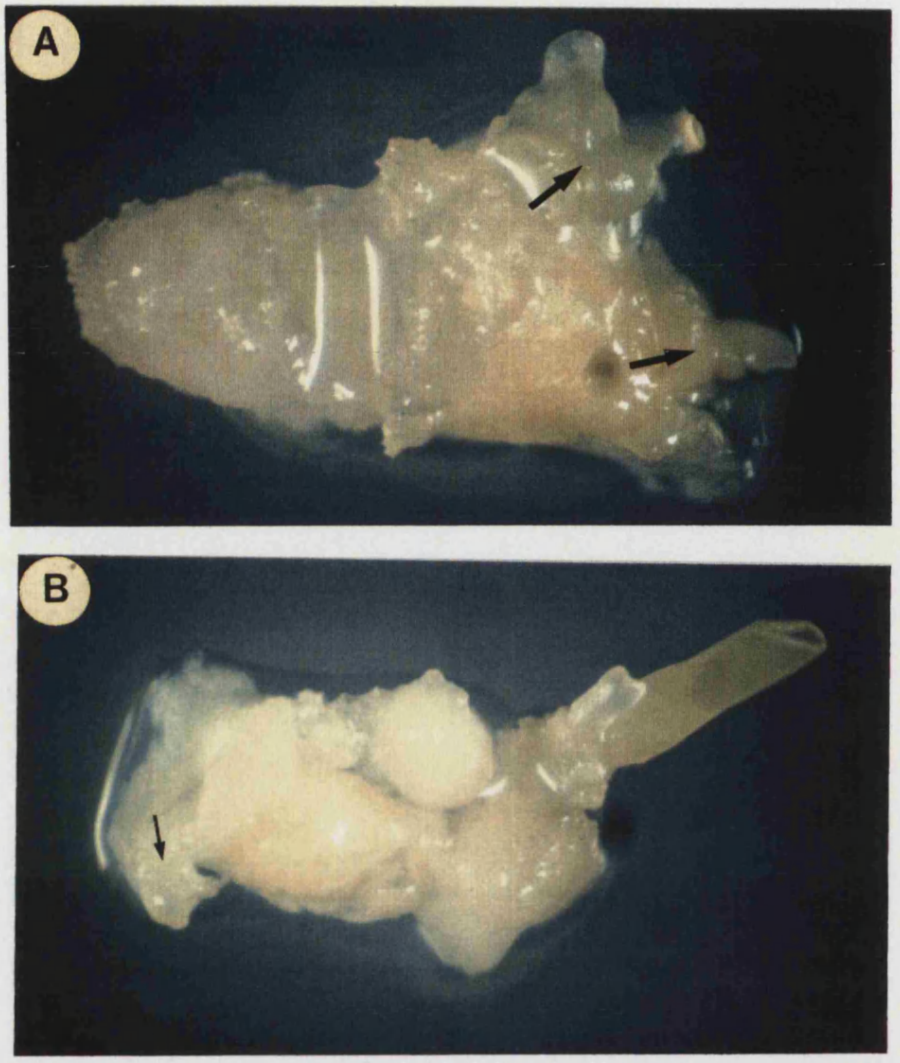
C
D
a
b
c
d
E

Plate 8.3.

Regeneration of frozen somatic embryos subjected to two stage cooling procedure

A and B. The growth of secondary somatic embryos produced by frozen somatic embryos. x25.

C. Regeneration of frozen somatic embryos cultured on different sucrose concentrations (5%, 7% and 9%) before and after freezing (see Section 8.3.5). x3.8.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best in terms of the production of secondary embryos since 50% of those embryos underwent embryogenesis.

Increasing the 2,4-D concentration from 0.01 mgl^{-1} to 0.05 mgl^{-1} caused an increase in the frequency of somatic embryos producing secondary embryos and the number surviving. Poorer results were obtained with 2,4-D concentration higher than 0.05 mgl^{-1}. The smaller embryos (0.5-1.0 mm) could not even survive when they were cultured on medium containing 2,4-D as high as 1.00 mgl^{-1} and were not able to produce secondary embryos on medium containing 2,4-D higher than 0.10 mgl^{-1}.

8.3.2. The effect of the duration of culture before freezing on the survival of frozen somatic embryos

An appropriate period of culture before freezing which allow the excised somatic embryos to recover without allowing them to grow larger was investigated by culturing the somatic embryos for either 3, 4 or 5 days. The medium used was MS semi solid medium supplemented with 0.05 mgl^{-1} 2,4-D and 2% (w/v) sucrose. Details of freezing procedures are described in Section 8.2. Plates 8.2A,C show the sizes of embryogenic tissues and somatic embryos used in cryopreservation.

Only somatic embryos that had been treated with 10% DMSO solution could survive irrespective of the period of culture before freezing (Table 8.4, Plate 8.2D). DMSO at 10% did not seem to affect the number of surviving unfrozen embryos, but the unfrozen embryos treated with DMSO produced more secondary embryos than the controls (without DMSO).

Table 8.4. Effect of duration of culture before freezing on the survival of frozen somatic embryos

	% DMSO (v/v)	Culture before freezing (days)	%embryos producing callus	%embryos producing secondary embryos	%embryos continuing growth	%total survival
Unfrozen	0	3	33.3	16.7	25.0	58.3
		4	25.0	33.3	83.3	91.7
		5	0	8.3	41.7	50.0
Frozen		3	0	0	0	0
		4	0	0	0	0
		5	0	0	0	0
Unfrozen	10	3	5.0	41.7	0	75.0
		4	41.7	41.7	1.7	91.7
		5	0	8.3	0	58.3
Frozen		3	0	0	0	0
		4	16.7	0	0	16.7
		5	8.3	0	0	8.3

Key :

Number of replicates : 12

Basal medium : MS supplemented with 0.05 mgl^{-1} 2,4-D

Duration of culture before freezing : 4 days

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\,\mu Mm^{-2}s^{-1}$ PAR

Cryopreservation procedure : I-stage : slow-cooled ($0.3^{o}Cmin^{-1}$) to $-30^{o}C$

II-stage : direct immersion in liquid nitrogen

Four days seemed to be the optimum duration for culturing somatic embryos before freezing on such medium in order to obtain the highest survival rate.

8.3.3. Investigations with cryoprotectants for use with somatic embryos

A range of DMSO concentrations was tested in order to find the optimum concentration which was not toxic and yet sufficiently high to protect the cells during freezing. Another experiment employing a combination of DMSO and glycerol was also carried out in conjunction with an investigation of the optimal period for cryoprotectant application.

8.3.3.1. The effect of DMSO concentration on the survival of frozen embryogenic tissues and somatic embryos

Both embryogenic and somatic embryos which had been cultured for four days were treated with either 5%, 10% or 15 % (v/v) DMSO for two hours before freezing. The medium used for culturing embryogenic tissues was as described in Section 8.3.1.2, while that used for culturing somatic embryos was supplemented with 0.05 mgl^{-1} 2,4-D and with either 2% or 7% (w/v) sucrose. Both embryogenic tissues and somatic embryos were subjected to freezing (see Section 8.2 for freezing details). Two weeks after freezing, the survival was assessed on the basis of embryo growth, new embryo production or callus production.

Table 8.5. The effect of DMSO concentration on the survival of frozen embryogenic tissues

Liquid nitrogen (-196°C)	% DMSO (v/v)	%embryos producing callus	%embryos producing secondary embryos	%embryos continuing growth	%total survival
Unfrozen	0	0	50.0	83.3	91.7
	5	0	25.0	75.0	91.7
	10	0	33.3	83.3	100.0
	15	0	50.0	91.7	91.7
Frozen	0	0	0	0	0
	5	0	0	0	0
	10	8.3	0	0	8.3
	15	50.0	25.0	0	50.0

Total χ^2 (survival = 7.4^{**}, with 2 degrees of freedom, giving $P>0.01$

Total χ^2 (secondary embryo production) = 9.6^{*}, with 2 degrees of freedom, giving $P>0.05$

Key :

Number of replicates : 12

Basal medium : MS supplemented with 0.01 mgl^{-1} 2,4-D and 2% (w/v) sucrose

Duration of culture before freezing : 4 days

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, $30\mu Mm^{-2}s^{-1}$ PAR

Cryopreservation procedure : I-stage : slow-cooled ($0.3^{o}Cmin^{-1}$) to $-30^{o}C$

II-stage : direct immersion in liquid nitrogen

Surviving embryogenic tissues could only be observed among those treated with either 10% or 15% (v/v) DMSO (8.33% and 50% survival rate respectively). Table 8.5 shows the details of these results. DMSO at 15% (v/v) applied to both embryogenic tissues and somatic embryos seemed to be superior to DMSO at 10% (v/v) if the medium used for culturing before freezing was supplemented with 2% (w/v) sucrose. When 7% (w/v) sucrose used in the medium, 10% (v/v) DMSO seemed to be the optimum concentration since 15% (v/v) DMSO gave rise to a higher frequency of frozen embryos producing callus only (Table 8.6).

DMSO at 5% (w/v) did not seem to be sufficient for cryopreserving cassava somatic embryos because at this concentration no survival could be obtained after freezing.

8.3.3.2. The effect of the concentration and duration of the application of DMSO and glycerol on the survival of frozen somatic embryos

In order to investigate the possibility of obtaining higher rates of survival, glycerol was also used as a cryoprotectant in combination with DMSO. In addition, the period of cryoprotectant application was increased from two hours, just before freezing, to four days plus a further two hours applied during the incubation before freezing (see Table 8.7). The procedures of cryoprotectant application and of freezing were described in Section 8.2.

10 % (v/v) DMSO applied either for two hours or for four days plus a further two hours prior to freezing gave rise to the highest rates of total survival (33.3%). Combining DMSO and glycerol resulted in a lower rates of survival regardless of the period of incubation (Table 8.7).

Table 8.6. The effect of concentration of DMSO on the survival of frozen somatic embryos cultured on different levels of sucrose before freezing

Liquid nitrogen (-196°C)	Sucrose level (gl^{-1})	% DMSO (v/v)	% embryos producing callus	% embryos producing secondary embryos	% embryos continuing growth	% total survival
unfrozen	20	0	0	8.3	66.7	75.0
		5	0	8.3	75.0	75.0
		10	16.7	25.0	41.7	83.3
		15	8.3	16.7	83.3	83.3
frozen		0	0	0	0	0
		5	0	0	0	0
		10	16.7	0	0	16.7
		15	25.0	8.3	0	25.0
unfrozen	70	0	0	8.3	25.0	33.3
		5	0	8.3	16.7	16.7
		10	0	8.3	8.3	16.7
		15	8.3	0	0	8.3
frozen		0	0	0	0	0
		5	0	0	0	0
		10	75.0	25.0	8.3	91.7
		15	83.3	25.0	0	91.7

Key : Total $\chi^2_{(survival)} = 14.9^{**}$, with 1 degree of freedom, giving P>0.01
$\chi^2_{(embryo\ production)} = 3.8^{*}$, with 1 degree of freedom, giving P>0.05

Number of replicates : 12

Basal medium : MS supplemented with 0.05 mgl^{-1} 2,4-D and 2% (w/v) sucrose

Temperature : 25±1°C

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

Cryopreservation procedure : I-stage : slow-cooled (0.3°Cmin^{-1}) to -30°C

II-stage : direct immersion in liquid nitrogen

Table 8.7. The effect of the concentration and duration of the application of DMSO and glycerol on the survival of frozen somatic embryos

	Cryoprotectant treatment				% survival of somatic embryos	% frozen somatic embryos producing secondary embryos
	DMSO (%v/v)		glycerol (%v/v)			
	4d+2h	2h	4d+2h	2h		
Unfrozen	-	-	-	-	50.0	41.7
	-	10.0	-	-	50.0	33.3
	5.0-10.0	-	-	-	50.0	50.0
	-	5.0	-	5.0	58.3	50.0
	2.5-5.0	-	2.5-5.0	-	58.3	50.0
Frozen	-	-	-	-	0	0
	-	10.0	-	-	33.3	16.7
	5.0-10.0	-	-	-	33.3	0
	-	5.0	-	5.0	25.0	0
	2.5-5.0	-	2.5-5.0	-	16.7	16.7

Key :

Number of replicates : 12

Basal medium : MS supplemented with 0.05 mgl^{-1} 2,4-D and 2% (w/v) sucrose

Temperature : $25 \pm 1^{o}C$

Light conditions : 16 hours photoperiod, 30 $\mu Mm^{-2}s^{-1}$ PAR

Cryopreservation procedure : I-stage : slow-cooled ($0.3^{o}Cmin^{-1}$) to $-30^{o}C$

II-stage : direct immersion in liquid nitrogen

The frequency of frozen embryos producing secondary embryos, however, seemed to be affected by the period of exposure to cryoprotectants. 10% (v/v) DMSO applied for two hours resulted in 16.7% of frozen embryos producing secondary embryos, while the longer exposure resulted in no secondary embryo production. In contrast, two hours exposure would not seem to be sufficient if DMSO was used in combination with glycerol since this did not result in any secondary embryo production, whereas 16.7% of frozen embryos were able to produce secondary embryos if this cryoprotectant combination was applied during the incubation before freezing, i.e. four days plus two hours.

8.3.3.3. The effect of the period of exposure to DMSO in combination with slow or rapid freezing on the survival of somatic embryos

Because of the ease of rapid freezing procedure (direct immersion in liquid nitrogen), this procedure was tested in comparison to a slow freezing procedure. The medium and culture conditions used were the same as those used in the previous experiments (MS + 0.05 mgl^{-1} 2,4D + 2% sucrose). 10% (v/v) DMSO was applied either for two hours only or for four days plus another two hours prior to freezing. Survival was assessed on the basis of embryo growth, embryo production and callus production.

There was no difference between slow cooled somatic embryos and those immersed directly in liquid nitrogen in terms of total survival (Table 8.8). All treatments brought about similar total rates of survival (33.3%) although those frozen directly in liquid nitrogen could only produce callus. Secondary embryos were only produced (16.67%) by embryos that had been treated with 10% (v/v) DMSO for two hours and slow cooled to -30^{o}C prior to immersion in liquid nitrogen (Table 8.8).

Table 8.8. The effect of the period of exposure to DMSO in combination with slow or rapid freezing on the survival of somatic embryos

Type of freezing	DMSO (%v/v)		% survival of somatic embryos	% frozen somatic embryos producing secondary embryos
	4d+2h	2h		
Unfrozen	-	-	50.0	41.7
	-	10.0	50.0	33.3
	5.0-10.0	-	50.0	50.0
slow	-	-	0	0
	-	10.0	33.3	16.7
	5.0-10.0	-	33.3	33.3
rapid	-	-	0	0
	-	10.0	33.3	0
	5.0-10.0	-	33.3	0

Key :

Number of replicates : 12

Basal medium : MS supplemented with 0.05 mgl^{-1} 2,4-D and 2% (w/v) sucrose

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, $30\mu Mm^{-2}s^{-1}$PAR

Cryopreservation procedure : slow-freezing : cooled to $-30^{o}C$ ($0.3^{o}Cmin^{-1}$)

rapid freezing: direct immersion in liquid nitrogen

8.3.4. The effect of a cool pre-treatment on the survival of somatic embryos cooled to different terminal temperatures

This experiment was carried out to investigate whether incubating the excised somatic embryos at 4°C instead of incubating them at 25°C for four days before freezing could increase the number of surviving embryos cooled to different terminal temperatures. Having been treated with 10% (v/v) DMSO for two hours, they were then cooled to different terminal temperatures : (i) cooled to -5°C ($12^{o}Cmin^{-1}$) and returned to room temperature, (ii) cooled to -5°C ($12^{o}min^{-1}$) and then ice crystallization was induced before rapid thawing to room temperature, (iii) cooled to -5°C ($12^{o}Cmin^{-1}$) and then ice crystallization was induced before further cooling ($0.3^{o}Cmin^{-1}$) to -30°C and finally rapid thawing to room temperature. The medium used and the culture conditions were similar to those used in Section 8.3.2.

The different cooling regimes led to different growth responses of the somatic embryos. Two weeks after cooling, 50% of somatic embryos that had been kept at 25°C and 44.7% of those that had been kept at 4°C before cooling to -5°C without ice formation had grown larger, while only 25% of those subjected to -5°C with ice formation and none of those subjected to -30°C had grown larger and formed large, dark green cotyledons (Table 8.9).

The highest frequency (41.67%) somatic embryos producing secondary embryos was obtained with somatic embryos incubated at 25°C, treated with 10% (v/v) DMSO for two hours and frozen to -30°C. 33.33% of those embryos cooled to -5°C with ice

Table 8.9. The effect of cold treatment and DMSO on the survival of slow-cooled somatic embryos

Pre-treatment temperature (°C)	Freezing temperatures	% DMSO (v/v)	%embryos producing callus	%embryos producing secondory embryos	%embryo continuing-growth	% total survival
25	Unfrozen	0	33.3	8.3	50.0	91.7
4		0	33.3	8.3	50.0	83.3
25	Unfrozen	10	5.0	41.7	41.7	91.7
4		10	33.3	16.7	25.0	66.7
25	-5°C without ice	0	25.0	0	0	25.0
4	formation	0	8.3	8.3	0	16.7
25	-5°C without ice	10	25.0	25.0	50.0	91.7
4	formation	10	16.7	25.0	41.7	66.7
25	-5°C with ice	0	8.3	0	0	8.3
4	formation	0	16.7	0	0	16.7
25	-5°C with ice	10	50.0	33.3	25.0	83.3
4	formation	10	75.0	25.0	25.0	91.7
25	-30°C with ice	0	0	0	0	0
4	formation	0	0	0	0	0
25	-30°C with ice	10	91.7	41.7	0	91.7
4	formation	10	66.7	25.0	0	75.0

Key : Total $\chi^2_{(survival} = 55.0^{**}$, with 2 degrees of freedom, giving P>0.01

Number of replicates : 12

Basal medium : MS supplemented with 0.05 mgl^{-1} 2,4-D and 2% (w/v) sucrose

Duration of culture before freezing : 4 days

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod, $30\mu Mm^{-2}s^{-1}$PAR

Cryopreservation procedure : I-stage : cooled to each terminal temperature ($0.3^{o}Cmin^{-1}$)

II-stage: immersion in liquid nitrogen

formation produced secondary embryos, while only 25% of those cooled to -5°C without ice formation gave rise to secondary embryos.

DMSO seemed to increase both total survival and the number of frozen somatic embryos producing secondary somatic embryos irrespective of the cooling temperatures. 25-41.7% of those embryos treated with 10% (v/v) DMSO produced secondary embryos, while 0-8.3% of the controls (frozen without DMSO) gave rise to secondary embryos.

Incubating the somatic embryos at 4°C for four days before freezing did not appear to be beneficial for the production of secondary embryos and the total number of survivors.

8.3.5. The effect of sucrose concentration on the survival of somatic embryos slow-cooled to -30°C or direct-cooled to -30°C prior to immersion in liquid nitrogen

Although some survival could be obtained in the previous experiment (Section 8.3.2), the frequency of survival including the frequency of frozen embryos producing secondary embryos was still quite low (16.7%). In an attempt to increase this frequency, therefore, an experiment employing different concentrations of sucrose (2%, 5%, 7%, 9%, 11% and 13%, w/v) added to the medium for culturing before and after freezing , was carried out. After freezing, the sucrose concentration was reduced by 2% steps to the standard concentration (2%) at three day intervals. The concentration and application of DMSO and the freezing procedures were as described in Section 8.2, except that some batches were cooled directly to -30°C prior to immersion in liquid nitrogen (Figure 8.2).

Table 8.10. The effect of sucrose concentrations and cooling regimes on the survival of frozen somatic embryos

Freezing procedure to -30°C	sucrose level (gl^{-1})	% DMSO (v/v)	%embryos producing callus	%embryos continuing growth	%embryos producing secondary embryos	%total survival
unfrozen	20	0	0	25.0	66.7	83.3
		10	16.7	25.0	41.7	66.7
slow		0	0	0	0	0
		10	16.7	0	0	16.7
rapid		0	0	0	0	0
		10	8.3	0	0	8.3
unfrozen	50	0	0	8.3	75.0	75.0
		10	8.3	16.7	50.0	66.7
slow		0	0	0	0	0
		10	33.3	0	0	33.3
rapid		0	0	0	0	0
		10	8.3	0	0	8.3
unfrozen	70	0	0	8.3	25.0	33.3
		10	0	8.3	8.3	16.7
slow		0	0	0	0	0
		10	75.0	25.0	0	91.7
rapid		0	0	0	0	0
		10	41.7	0	0	41.7
unfrozen	90	0	0	8.3	8.3	16.7
		10	25.0	0	0	25.0
slow		0	0	0	0	0
		10	66.7	8.3	0	66.7
rapid		0	0	0	0	0
		10	16.7	0	0	16.7

Table 8.10.1 (continued)

Freezing procedure to -30°C	sucrose level (gl^{-1})	% DMSO (v/v)	%embryos producing callus	%embryos continuing growth	%embryos producing secondary embryos	%total survival
unfrozen	110	0	8.3	0	8.3	16.7
		10	0	8.3	0	8.3
slow		0	8.3	0	0	8.3
		10	25.0	0	0	25.0
rapid		0	8.3	0	0	8.3
		10	8.3	0	0	8.3
unfrozen	130	0	0	16.7	0	16.7
		10	0	8.3	0	8.3
slow		0	8.3	0	0	8.3
		10	50.0	0	0	50.0
rapid		0	0	0	0	0
		10	8.3	0	0	8.3

Total $\chi = 19.2^{**}$, with 5 degrees of freedom, giving $P>0.01$

Key :

Number of replicates : 12

Basal medium : MS supplemented with 0.05 mgl^{-1} 2,4-D

Duration of culture before freezing : 4 days

Temperature : $25\pm1^{o}C$

Light conditions : 16 hours photoperiod,

$30\mu Mm^{-2}s^{-1}$ PAR

Cryopreservation procedure : slow freezing : cooled to $-30^{o}C$ ($0.3^{o}Cmin^{-1}$) prior to immersion in liquid nitrogen

rapid freezing: direct immersion in liquid nitrogen

Increasing the sucrose concentration from 2% to 7% (w/v) in combination with both the slow- cool to -30^{o}C and direct-cool to -30^{o}C procedures resulted in an increase in survival and production of secondary embryos, while the use of concentrations higher than 7% (w/v) resulted in decreased survival. Sucrose at 7% (w/v), therefore, resulted in the highest frequency of somatic embryos surviving (91.7%) and producing secondary embryos (25.0%). Plates 8.2D-8.3C show the regeneration of frozen somatic embryos.

Survival rates of up to 50.0% and 8.3% (slow-cooled and direct- cooled to -30^{o}C respectively) could still be obtained with 13% (w/v) sucrose in the medium but the embryos were only able to produce callus (Table 8.10). Without the application of DMSO, this sucrose concentration also resulted in 8.3% survival.

Besides producing a lower survival rate, direct- cooling to -30^{o}C only allowed the production of friable callus without secondary embryos.

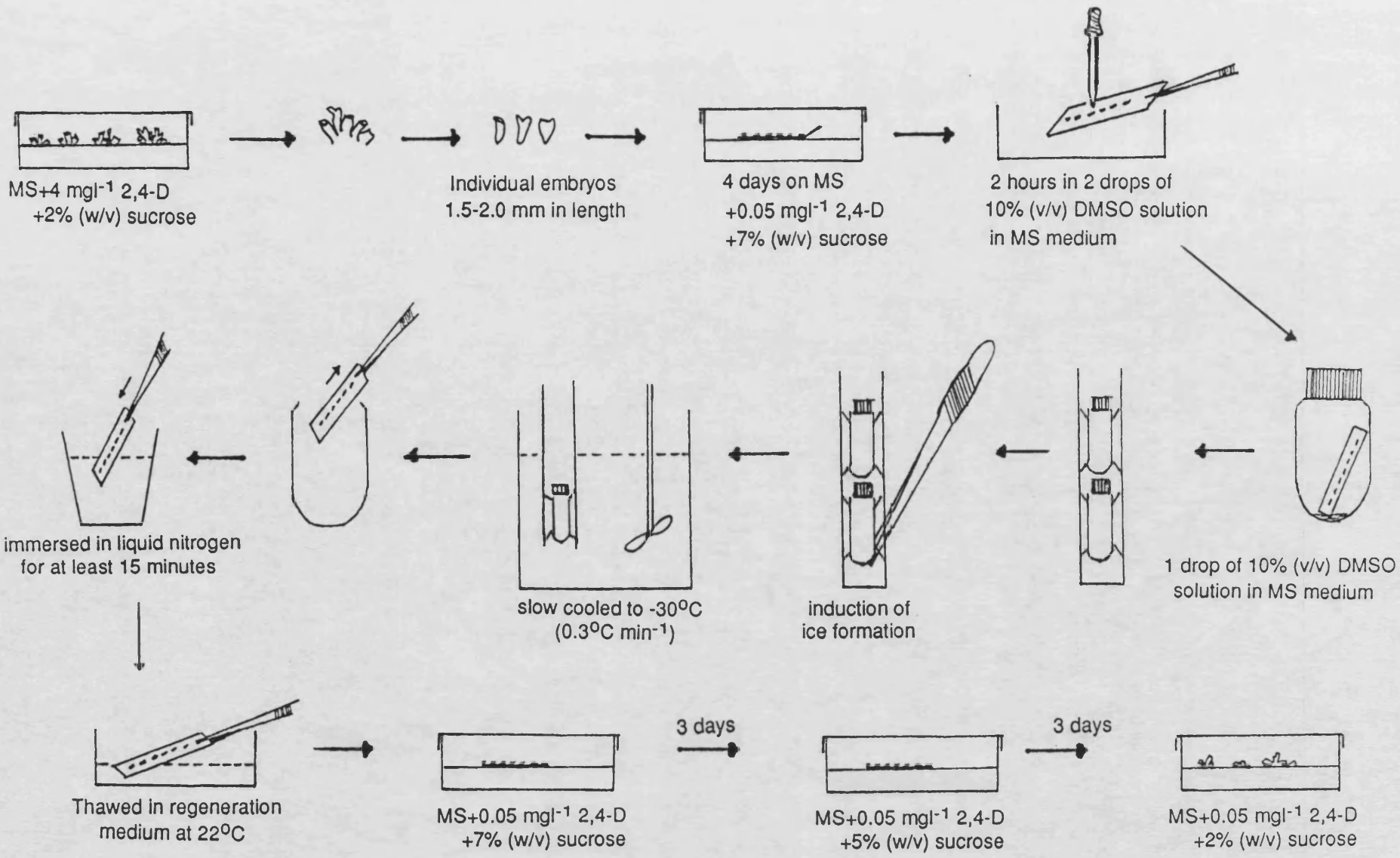

Fig. 8.2. Summary of procedure for cryopreservation of cassava somatic embryos

8.4. DISCUSSION

The preliminary experiments were aimed at identifying the type of embryogenic explant that would be most suitable for use with the cryopreservation techniques. It was assumed that such an explant, as well as being as small as possible in overall size, should contain a high proportion of small, densely cytoplasmic cells and that it should be capable of responding vigorously in an organized manner when sub-cultured.

The results (Section 8.3.1.1) indicated that only the lower parts of somatic embryos were not able to produce secondary embryos. It would also seem that there was no further growth of this part of somatic embryos during the culture incubation. This was, presumably because the dominant meristematic region, although not clearly distinguishable, was located in the top part of somatic embryo above the hypocotyl.

It would seem that there is a strong relationship between the concentration of 2,4-D and the stage of development; the more organized somatic embryos seem to need a higher concentration of 2,4-D than the less organized embryogenic tissue which is more sensitive. It would also seem that the smaller the size of organized tissue, the lower the concentration of 2,4-D needed for continued development. As reported in Section 8.3.1.2 only somatic embryos in a range of size 1.5-2.0 mm and 2.0-2.5 mm in length were able to survive and produce secondary embryos (10% and 20% respectively) when they were cultured on medium supplemented with 5 mgl^{-1} 2,4-D. Culturing those embryos on medium with lower concentration of 2,4-D (0.05 mg^{-1}) resulted in the

highest frequency of somatic embryos producing secondary embryos (50% and 40% respectively). This range of size is, however, rather larger than that of somatic embryos of *Citrus* successfully used for cryopreservation i.e. 0.5-1.0 mm in length (Marin and Duran-villa, 1988).

As well as establishing the appropriate explant and suitable culture conditions, it was also important to establish the optimal period for culture incubation prior to freezing. Withers (1984) stated that the success of cryopreservation would not be assured unless the cultures were grown before cryopreservation under conditions which allow them to develop the maximum content of cytoplasm and minimum cell size. Four days, which appeared to be the optimum for cassava (Section 8.3.2), would seem to be in the range reported for somatic embryos of other plant species; somatic embryos of oil palm required seven days incubation before they were frozen (Engelmann and Dereudree, 1988), while those of citrus (Marin and Duran-villa, 1988) required only one to three days.

During the incubation before freezing, cryoprotectant solution has also been applied by some investigators such as Kartha *et.al.*(1979) in work with pea meristems. In the case of cassava somatic embryos, the application of DMSO at 10% (v/v) during culture incubation before freezing decreased the frequency of frozen somatic embryos producing secondary embryo from 16.7% to 0% (Section 8.3.3.2) and it appeared that DMSO at 10% (v/v) was toxic if it was applied for longer than two hours; therefore a lower concentration of DMSO either alone or in combination with another cryoprotectant such as glycerol might be required if longer exposure were to be carried out. However, because of the superiority of DMSO applied alone at a concentration of 10% (v/v) two hours before freezing, this treatment was chosen for the further work (Section 8.3.3.1). This result supported Meryman and Williams' conclusion (1981) in that for many

applications, DMSO will be preferable because of its penetration into most cells so that it can protect against cell-volume loss over and above its pure colligate effects.

The investigations into the cryoprotectant effects of sucrose in combination with 10% (v/v) DMSO showed that an increase in the sucrose concentration from 2% to 7% (w/v) resulted in an increase in the survival rate of cassava somatic embryos from 16.7% to 91.7% and the frequency of secondary embryo production from 0 to 25%. This was in accord with the report of the beneficial effects of sucrose as a cryoprotectant in combination with DMSO with somatic embryos of oil palm (Marin and Duran-villa, 1988) and of citrus (Engelmann and Dereudree, 1988). Some survival (8.3%) of cassava somatic embryos was also obtained with high levels of sucrose (11% and 13%, w/v) were used in the absence of DMSO. This result showed some similarity to the results of Orr *et.al.* (1986) showing that the presence 13% sucrose alone induced a similar level of hardening as 6.5% sucrose plus ABA to obtain a higher level of freezing tolerance of meristems of *Brassica napus.*

Another attempt to increase the survival rate involved in the incubation of somatic embryos in a cold room (4^{o}C) for four days prior to freezing (Section 8.3.4). Unlike the results with other plant species obtained by some workers, incubating cassava somatic embryos in a cold room lowered the frequency of survival from 91.7% to 66.7%. Sakai and Sagawari (1973) in work with *Populus euramericana*, and Seibert and Wetherbee (1977) in work with *Dianthus caryophyllus* achieved a marked improvement in the survival of frozen tissues by the application of cold hardening treatment. It would seem, therefore, that cold treatment was not applicable to all plant species. Possibly the recovery and growth of cassava somatic embryos after excision was inhibited by the low temperature or possibly the lower temperatures does not induce a hardening effect in a tropical species such as cassava.

The results from Section 8.3.4 also showed that the terminal temperatures to which the somatic embryos were cooled during the first-stage cooling were also likely to affect their subsequent growth after cryopreservation. The lower the temperature to which the somatic embryos were cooled or frozen, the lower the frequency of embryos that grew larger and the higher the frequency of embryos that produced callus and secondary embryos. Thus, slow-cooling the somatic embryos to -30^{o}C brought about the highest frequency of frozen somatic embryos producing secondary embryos. If the somatic embryos were rapidly cooled to -30^{o}C, however, this frequency was decreased to 0% and only callus was produced. Rapid-cooling to -30^{o}C, therefore, would seem to cause a loss of organization which resulted in only callus being produced (Section 8.3.5). This could also explain why direct immersion in liquid nitrogen (-196^{o}C) also led to the production of callus from somatic embryos (Section 8.3.3.3). These freezing methods, according to Withers (1978) cause inadequate dehydration, excessive supercooling and a large amount of intracellular ice.

CHAPTER 9

FINAL DISCUSSION

The first aim of this study was to induce continuous proliferation of somatic embryos from a wider range of cassava cultivars at a high frequency. Both immature leaf lobes and axillary buds from clonal plants in the greenhouse and from *in vitro* plantlets of five CIAT cultivars, and those of shoot-derived plantlet *in vitro* of 15 IITA cultivars (Sections 3.3.1.1-3.3.1.2) could undergo somatic embryogenesis at a high frequency (varying between 10% and 100%, depending on cultivars). One IITA cultivar failed to produce somatic embryos, but it was only tested with 2,4-D. Although both axillary buds and cotyledon from seeds were able to produce somatic embryos under suitable conditions, leaf lobes were used routinely throughout this study due to the fact that they produced more somatic embryos than the axillary buds, and they required a shorter incubation period in the 2,4-D medium than the cotyledon explants.

Leaf lobes explants have been used as well as cotyledons in earlier work (Stamp and Henshaw, 1981); however, the frequency of leaf lobes particularly those taken from clonal plants in the greenhouse, producing somatic embryos was low (up to 29%). Moreover, embryogenesis was restricted to a limited number of cultivars and it required longer periods of incubation in Stage-I medium (24-31 days) to achieve the highest frequency of response. The results here (Sections 3.3.1.1.3 and 3.3.1.2.1) showed that a higher frequency of embryogenesis (up to 80% of leaf lobes from clonal plants and 100% of those from plantlets *in vitro*) could be obtained by culturing leaf lobes for 14 days on medium supplemented with 4 mgl^{-1} 2,4-D. One factor which might be responsible for the increase of this frequency is the age of the clonal plants in the greenhouse from which the leaf lobes were taken. Leaf lobes taken from 4 month-old clonal plants grown in the greenhouse resulted in a higher frequency of embryogenesis and total production of somatic embryos than that of 2 month-old clonal plants (Section 3.3.1.6) Moreover it was shown that the effect of the size of leaf lobes used as explants was important in terms of the production of somatic embryos; leaf lobes of 3-5 mm size

produced the highest frequency of embryogenesis in most experiments, they were significantly better than those of 5-7 mm size (Section 3.3.1.1.1). It was also shown that the periods of incubation required by the different sizes of leaf lobes were significantly different; larger leaf lobes would seem to require longer incubation in medium with a higher concentration of 2,4-D (Section 3.3.1.1.1). The effect of the physiological state of explants on somatic embryogenesis has been reported by several investigators. Banks (1979) reported that only cultures initiated from adult material of English ivy (*Hedera helix*) produced somatic embryos. In the Gramineae, the developmental stage of the explant was found to be the most critical factor in obtaining the optimal response and in establishment of vigorously growing embryogenic tissues (Vasil, 1982). Immature inflorescences in which primordia of individual floral organs had just begun to be formed was proved to be the best explant for the induction of embryogenesis in *Pennisetum americanum* (Botty and Vasil, 1984), *Sorghum arundinaceum* (Boyes and Vasil, 1984), *Panicum miliaceum* (Rangan and Vasil, 1983).

In addition to 2,4-D, picloram and dicamba were shown to be capable of inducing somatic embryogenesis in cassava. The positive effect of 2,4-D was restricted to certain CIAT cultivars such as CMC 76, MCol 22 and MCol 216, but the leaf lobes of recalcitrant cultivars such as CMC 40 and MCol 113 could respond to medium supplemented with either picloram or dicamba, although the production of somatic embryos was significantly lower than with CMC 76 which was the most responsive cultivar with 2,4-D. It was also shown that the recalcitrant cultivars, particularly CMC 40, required longer exposure to the picloram medium, since 30 days incubation in this medium significantly increased the production of somatic embryos (Section 3.3.1.2.1). The ability of picloram and dicamba to induce embryogenesis has been reported in other plant species. A low concentration of picloram (0.06 mgl^{-1}) was successfully used to induce somatic embryos from *Pisum sativum* (Jacobsen and Kysely, 1984), *Gasteria* and *Haworthia* (Beyl and Sharma, 1983)

and from leaf-derived callus of pea Jacobsen and Kysely, 1984) upon transfer to liquid medium. In *Vinga mungo*, picloram (0.5 mgl^{-1}) was also able to induce somatic embryos from mesophyll protoplasts (Sinha *et al.*, 1984). Dicamba at a concentration of 30 µM in SH medium has been successfully used for the induction of *Zea mays* embryogenic tissues (Conger *et al.*, 1987), while a concentration of 10-20 µM in MS medium was used to induce banana somatic embryos (Litz, 1988).

The optimum concentration of picloram and dicamba which could induce somatic embryogenesis varied depending on the cultivars used. Leaf lobes of CMC 76, which was considered to be the most responsive cultivar to 2,4-D, required a lower concentration of picloram and dicamba than other cultivars (Section 3.3.1.2). As both the frequency and productivity of embryogenesis with cultivar CMC 76 were high, this cultivar was used for most subsequent experiments including those involving cell and protoplast cultures. Productivity, which was defined as the number of somatic embryos produced per explant would seem to be an important parameter for describing the response of plants to embryogenesis. Since, in some situations, the frequency of explants producing somatic embryos could be high, whilst the productivity was low.

Attempts to induce somatic embryogenesis from single cells isolated from leaves either mechanically or enzymically were unsuccessful. Difficulties were encountered in isolating single cells from leaves with the result that it was only possible to use relatively low inoculum densities (Section 5.3.2). Alternative sources of single cells, such as suspension cultures and somatic embryos cultured on solid medium, might be used to overcome this problem, particularly since somatic embryos subsequently proved to be a good source of protoplasts of cassava (Section 5.3.3). Bidney and Shepard (1980) showed that both single cells and protoplasts could be obtained by macerating leaves and petioles of sweet potato enzymically and by varying the types and combinations of

enzymes a higher proportion of single cells could be obtained. The same type of enzyme has been shown to give different effects when the enzyme was obtained from different sources. Macerozyme produced by Yakult Biochemicals Co. was superior to Macerozyme R-10 produced by Kinki Yakult Mfg. Co. Ltd in terms of cell release (Bhojwani and Razdan, 1983).

A different medium and certain culture techniques were required to initiate cell division and the formation of embryogenic cells in single cell cultures, since the medium used by Shahin and Shepard (1980) did not give a satisfactory results. Adding different type of cytokinin such as zeatin, and some macro nutrients might result in a better results. In carrot, the addition of medium supplemented with 10^{-8} M zeatin and 15 mM $CaCl_2$ to the medium containing 5 x 10^{-7} M 2,4-D was necessary to increase the number of spherical cells for the induction of embryogenesis from single cells (Nomura and Komamine, 1985). In the population, as well as spherical cells which had a diameter about 12 um and differentiate to embryos at a frequency of about 90%, two other cell types were also distinguished namely : oval shape cells which frequency of differentiation was only about 10%, and elongated cells which did not differentiate to embryos at all under the condition used. It was necessary to eliminate those two types of cells particularly those which could not differentiate to embryos for high frequency of embryogenesis.

As a good source of protoplasts, somatic embryos seem to offer advantages over other sources such as leaf explants, since it has been reported in other plant species that the yield and the capacity to divide of both single cells and protoplasts from leaves seemed to be affected by the stage and the physiological state of the clonal plants. In pea (Jia, 1982) the percentage survival of protoplasts from lower leaves was much

higher than that of protoplasts from the uppermost leaves, but in contrast, cell division only occurred in protoplasts from the uppermost or next to the uppermost expanded leaf. Apparently, as the leaves became older, the tolerance of the cells to the enzymic digestion was increased, but the capacity to divide was decreased. The effect of the physiological state of plants grown in the greenhouse on the success of protoplast culture has also been reported in *Brassica oleracea* and *Brassica campestris* (Schwenk and Hoffmann, 1979). Mesophyll protoplasts of these species isolated from plants grown in a greenhouse or growth cabinet failed to divide but those from aseptically growing shoots could form calli.

The results showed that cassava protoplasts required high concentrations of 2,4-D, since medium with 2.0 mgl^{-1} 2,4-D in combination with NAA and BAP resulted in better development in terms of cell wall formation than medium containing 0.2 mgl^{-1} 2,4-D. A high concentration of 2,4-D (2.0 and 5.0 mgl^{-1}) has also been used in wheat (Harris *et al.*, 1989) and in *Primula malacoides* (Mii, 1990). In future work, therefore, it would probably be advisable to employ a more complex medium containing a relatively high concentration of 2,4-D (2.0 to 4.0 mgl^{-1}) in combination with NAA and BAP to induce division and better growth. The addition of NAA to a medium containing a combination of 2,4-D and BAP has been successfully used for culturing protoplasts of other plant species such as melon (Bokelmann, *et al.*, 1990) and *Primula malacoides* (Mii, 1990). A more complex medium is apparently required, since standard MS medium did not seem to give good results (Section 5.3.3). The addition of certain macro nutrients such as KNO_3, $CaCl_2.2H_2O$, and KH_2PO_4, of amino acids and a complex mixture such as coconut water and the use of glucose instead of mannitol or a combination of both osmoticum agents might be a useful approach in further studies. Levels of macro nutrients particularly KNO_3 and KH_2PO_4 up to four times higher than those in MS medium have been used to culture *Solanum* sp. (Haberlach *et al.*, 1985) and *Vinga mungo*

(Sinha, *et al.*, 1983) protoplasts. Coconut water and glutamine have been used to favour the develoment of protoplasts of barley and wheat (Lazzeri and Lorz, 1990 and Vasil *et al.*, 1990 respectively) and of wheat (Farmer and Lee, 1977) respectively. Atanasov and Brown (1984) used a complex medium supplemented with glucose, penicillin and streptomycin sulfate to support cell division and colony formation of *Medicago sativa* protoplasts. The addition of glucose in combination with mannitol has been reported to be beneficial for protoplast culture of melon (Bokelmann *et al.*, 1990) and of barley (Lazzeri and Lorz, 1990).

In addition to the composition of the medium, a lower initial density of protoplasts, different culture techniques and culture conditions would probably be needed improve cassava protoplast cultures. Since the lowest density tested (2×10^5 protoplasts per ml) resulted in the best protoplast development (Section 5.3.3), lower densities in the range of 10^4 - 10^5 protoplasts per ml cultured with different gelling agents such as gelrite and incubated at higher temperatures such as 27-28^{0}C might give better results. Gelrite was shown to be the best gelling agent for protoplast cultures of melon (Bokelmann *et al.*, 1990) and of *Primula malacoides* which were cultured on discs of gelrite placed in 2 ml of liquid medium (Mii, 1990). Higher temperatures (27-29^{0}C) have proved beneficial for cell division in protoplast cultures of *Lycopersicum esculentum* and of *Lycopersicum peruvianum* (Zapata *et al.*, 1977) and of *Gossypium hirsutum* (Bhojwani *et al.*, 1977).

The establishment of cassava suspension cultures from which somatic embryos could be regenerated apparently depended upon several factors such as the type of initial tissues, the shaking rate, the concentration of 2,4-D and the subculture regime (Section 5.3.1). As with the induction of embryogenesis on solid medium, 2,4-D at 4 mgl^{-1} would also seem to be the optimum concentration for the establishment of embryogenic cell suspension cultures (Section 5.3.1.1). Only embryogenic tissues

cultured in this medium rotated at 120 rpm could initiate embryogenic suspension cultures. Similar conditions involving medium containing 2,4-D and shaking rate have also been used to initiate embryogenic suspension cultures of daylily (Krikorian and Kann, 1981), and of *Phaseolus acutifolius* and *Vigna aconitifolia* (Kumar *et al.*, 1988 a,b).

Plating the suspension cultures on semi-solid medium appeared to be necessary to allow the embryogenic clumps to develop further and become mature embryos. Different levels of 2,4-D used for the initiation and maintenance of suspension cultures led to a different morphogenetic response: a high frequency of root formation was observed from cultures with 2,4-D at a concentration of 8 mgl^{-1}. In Gramineae, some suspension cultures often form only roots, but sometimes also somatic embryos which may develop to maturity and are later released into the medium. However, only a small number are able to develop to maturity since the majority of them undergo secondary callusing or germinate precociously to give rise to weak plantlets (Vasil, 1982). The types and concentration of hormones used in the medium for plating the embryogenic clumps seemed to affect the frequency of further embryogenesis. Medium with a combination of NAA and BAP could result in further embryogenesis although the frequency was lower than that on 2,4-D medium (Section 5.3.1.3).

Due to the fact that most cassava somatic embryos produced in suspension cultures were in clumps (Section 5.3.1) which required separation following transfer on solid medium, it might be useful to investigate media or conditions that would increase the number of individual embryos in the population. Nadel *et al.*(1989) demonstrated that the addition of mannitol (3-4%, w/v) prevented cell lysis, greatly increased the number of single somatic embryos formed, improved the normal differentiation of embryos, and accelerated torpedo embryo development.

Somatic embryos obtained from explants grown either on solid or liquid medium or obtained from embryogenic suspension cultures were able to regenerate to plantlets. However, it was noted that the frequency of normal plantlets regenerated from primary somatic embryos was no higher than the 33.3% obtained with somatic embryos 1-3 mm in size (Section 7.3.1). This was presumably due to cell abnormalities induced when the somatic embryos were still in clumps. The development of the shoot apex can be affected by changes in the sequence of cell division, enlargement and maturation; for example it has been suggested that increased cell division and enlargement during the initiation of cotyledons can generate an enlarged apical region (Ammirato, 1982). In somatic embryos, the embryogenic shoot apex may continue its development to form a seedling shoot apex leading to precocious germination. This premature differentiation may occur when the cotyledons are initiated forming a broad, flat, non-functioning shoot apex which was observed in somatic embryos of caraway (Ammirato, 1985) and of soya bean (Lazzeri *et al.*, 1987). This development possibly occurred from tubular shape cotyledons of mature cassava somatic embryos which scanning electron micrograph showed forming broadly flattened and enlarged shoot apices.

It has been shown that the normality of somatic embryos might be improved by the use of ABA. Ammirato (1974) showed that in caraway the effect of ABA on the external morphology of the somatic embryos was quite specific to the times application. If ABA was added at the time that the proembryo suspension was transferred to maturation medium, predominantly single embryos with two cotyledons were produced, whereas adding ABA at one week old cultures led to a substantial increase in the production of multiple embryos and those with accessory embryos. With cassava, adding 2.0 mgl^{-1} ABA to the 2,4-D medium used for the induction of secondary embryos from primary embryos led to the production of more uniform shape of secondary embryogenic tissues which eventually regenerated to normal somatic embryos (Section 4.3.2). It would be

recommended, therefore, to use ABA routinely in combination with 2,4-D to obtain uniform development of secondary embryos.

Another factor that might have caused not all of somatic embryos being able to regenerate to normal plantlets is the fact that the stage of development of somatic embryos in the clumps was highly variable. When they were separated individually and placed on regeneration medium, some somatic embryos which were still very small did not develop further and eventually died; in contrast, some somatic embryos already possessing large dark green cotyledons which were normal in appearance failed to produce a normal shoot. There have been many reports in the literature of apparently well-formed somatic embryos that elongate but fail to produce plantlets. it has been suggested that this most likely can be attributed to the malformation of the shoot apical meristem (Kerns, *et al.*, 1986). To overcome the variability resulting from the stage of development, sequential separation according to the development could be practised, so that only somatic embryos with the optimal size (1-3 mm) are separated, while smaller embryos are left to develop for a longer period. Another strategy to increase the frequency of normal plantlets could be based on the optimization of the period on the regeneration medium so that the highest proportion of 1-3 mm somatic embryos is produced before they are separated individually.

A higher frequency of normal plantlets (84.4%) could be obtained by culturing secondary embryos individually on regeneration medium (Section 7.3.1). The superiority of secondary embryos is possibly due to the fact that they were more uniform in terms of size or development than primary somatic embryos. This uniformity is apparently due to the removal of mature embryos and friable callus during secondary embryo proliferation. Another advantage of culturing secondary embryos on regeneration medium is that they

naturally posses tap root, while the frequency of primary somatic embryos possessing tap root is very low.

This properties of secondary embryos, together with the fact that the productivity of primary embryos with regard to embryo production was 10-12 fold higher than productivity of leaf lobes taken from the plant, led to the investigation of methods for the continuous proliferation of secondary embryos. A successful procedure that was established for the continued proliferation of somatic embryos involved alternating passages on semi-solid MS medium containing respectively 2.0 and 4.0 mgl^{-1} 2,4-D. As an alternative, clumps of somatic embryos could be maintained by culturing them on semi-solid MS medium with a combination of 4.0 mgl^{-1} 2,4-D and 2.0-4.0 mgl^{-1} ABA (Section 4.3.2). Long exposure (40 days) on medium with a high concentration of ABA (4.0 mgl^{-1}) caused water-logging, although these somatic embryos recovered and regained their embryogenic potential following tranfer to hormone-free medium. Qureshi *et al.*(1989) provided strong evidence in support of the physiological availability of a certain level of ABA for the prevention of precocious germination and maintenance of embryogenic capacity in wheat. A lower level of ABA (0.10-0.25 mgl^{-1}) in the medium supported an embryogenic response from the cultured embryos but fewer somatic embryos were produced and they germinated precociously. On the other hand, the presence of 0.50 mgl^{-1} ABA in the medium produced a higher embryogenic response and prevented precocious germination of somatic embryos.

The procedure for the induction and continued proliferation of secondary embryos is of considerable importance, since it now provides a system capable of producing large numbers of embryos which can be regenerated to normal plants with high frequency. Chromosome counts with the somatic embryos and the regenerated plantlets did not reveal any changes in chromosome numbers and, although this is not proof of total

genetic stability, it is in agreement with the results obtained by Hanna *et al.*(1984) and Swedlund and Vasil (1984) with plants regenerated from somatic embryos of *Panicum maximum* and *Pennisetum americanum.*

Perhaps more significantly, the embryogenic system can be used to produce supply of viable protoplasts (Section 5.3.3) and at present, it is the only reliable regeneration procedure which might be contemplated for use as part of a genetic transformation system.

A long-term storage procedure for embryogenic tissues and somatic embryos of cassava under what are believed to be genetically stable conditions, was also established. This involved cryopreservation in liquid nitrogen following a slow-cooling sequence ($0.3^{o}Cmin^{-1}$) to $-30^{o}C$. The size of embryogenic tissues and somatic embryos, the concentration of 2,4-D and of sucrose in the medium used before and after freezing, the culture conditions, and the type and concentration of cryoprotectant, were all important factors in the design of the successful protocols (Section 8.3).

The choice of cultures to be frozen has a significant effect on recovery rate. It is known that there is a close relation between growth cycle stage of cells and their survival potential (Nag and Street, 1975; Withers, 1978, 1984); for example, the relatively small cells with a high cytoplasmic content that are characteristic of the late lag phase or early exponential phase in suspension cultures have the highest freeze tolerance. The physiological state and morphology of the cells , particularly cell size and degree of vacuolation, therefore clearly affect survival in the freezing-thawing procedures. Within any one species, cells at minimum size, e.g., in exponential growth or in a meristematic zone are more likely to survive. The location of cells within a multicellular structure can also affect their survival, with inner cells tending to be more susceptible to damage than

superficial ones. A culture of small aggregate size is at a double advantage, since it has a higher proportion of superficial cells and dehydration/rehydration stresses due to aggregate size are minimal. It is, therefore, important to use cells grown under conditions where they develop maximum content of cytoplasm and minimum size. Small embryogenic clumps and young somatic embryos might have been expected to satisfy these criteria and this was confirmed in practice.

The addition of DMSO and the interaction between DMSO and sucrose were shown to increase significantly the survival of frozen somatic embryos (Section 8.3.5). In addition, DMSO added to the medium for culturing unfrozen somatic embryos seemed to stimulate secondary embryogenesis. The effect of DMSO on the induction of secondary embryogenesis has also been noted by Qureshi *et al.*(1989), who showed that increasing the DMSO concentration up to 0.2% (v/v) in the induction medium increased the percentage of embryos producing embryogenic callus. Despite the fact that sucrose alone did not significantly increase the total survival of frozen somatic embryos, high concentration of sucrose (11 and 13%) led to 8.33% of the controls (frozen without DMSO) surviving.

Although it has been shown that somatic embryogenesis could be induced from a wide range of cassava cultivars on semi-solid medium, plant regeneration from somatic embryos could be obtained at a high frequency, embryogenic suspension cultures could be established, and viable protoplasts could be isolated, further studies involving embryogenesis on semi-solid medium and suspension and protoplast cultures are of considerable important. It would be useful if other cassava cultivars which are capable of undergoing embryogenesis (Sections 3.3.1.1.3 and 3.3.1.2) could also widely used in other experiments such as for the establishment of suspension cultures and for protoplast cultures, since only CMC 76 which was the most responsive cultivar used for

most subsequent experiments. Continuous somatic embryo proliferation, maintenance of somatic embryos and the possibility of preserving somatic embryos in liquid nitrogen, of those cultivars also require further investigations.

Embryogenic system is now being used as an essential component of a genetic transformation procedure aimed at the enhancement of virus resistance in cassava (Fauquet and Schopke, pers. comm.). At present this is the only *in vitro* regeneration system in cassava that has the potential for use in genetic transformation procedures and it is now important that it should be further developed so that it works efficiently with a wider range of genotypes. In addition, studies of regeneration from transformed tissues are of great important, since they might require some modifications in terms of the composition of medium and culture conditions.

An improved suspension cultures producing a large amount of individual somatic embryos floating freely in the medium would support the realisation of coating cassava somatic embryos as "artificial seeds". The ability to maintain somatic embryos using ABA and to cryopreserve somatic embryos in liquid nitrogen open the possibility to encapsulate cassava somatic embryos as an "artificial seed". Different types of auxin used either alone or in combination with 2,4-D, and the use of ABA routinely in suspension cultures would be useful investigations. Kitto and Janick (1985) investigated the influence of ABA on subsequent survival of encapsulated somatic embryos of carrot. They demonstrated that treated carrot somatic embryos with 10^{-7} or 10^{-6} M ABA during two week embryo induction phase in an effort to block precocious germination has increased the survival to 40% during encapsulation. Like seed, encapsulated somatic embryos must retain a minimum moisture content in order to remain viable. Increased survival of encapsulated ABA-treated carrot somatic embryos may be due to the

imposition of a developmental arrest (quiescene) during which time embryos mature and develop desiccation resistance (Kitto and Janick, 1985).

More single cell and protoplast work would certainly be necessary to achieve cell division, embryogenesis and plant regeneration.

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Appendix I. Composition of Murashige and Skoog basal medium (Murashige and Skoog, 1962).

Macronutrients	mgl^{-1}	mM
NH_4NO_3	1650	20.60
KNO_3	1900	18.80
$CaCl_2.2H_2O$	440	3.00
$MgSO_4.7H_2O$	370	1.50
KH_2PO_4	170	1.25
Micronutrients		**uM**
KI	0.83	5.00
H_3BO_3	6.20	100.00
$MnSO_4.4H_2O$	22.30	100.00
$ZnSO_4.7H_2O$	8.60	30.00
$NaMoO_4.2H_2O$	0.25	1.00
$CuSO_4.5H_2O$	0.025	0.10
$CoCl_2.6H_2O$	0.025	0.10
Na_2.EDTA	37.30	100.00
$FeSO_4.7H_2O$	27.80	100.00
Vitamins		
Inositol	100.00	
Nicotinic acid	0.50	
Pyridoxine.HCl	0.50	
Thiamine.HCl	0.10	
Glycine	2.00	

Appendix II. Composition of B5 basal medium (after Gamborg, *et al.*, 1968)

Macronutrients	**mgl^{-1}**	**mM**
KNO_3	2500	25.00
$CaCl_2.2H_2O$	150	1.00
$MgSO_4.7H_2O$	250	1.00
$(NH_4)_2SO_4$	134	1.10
$NaH_2PO_4.H_2O$	150	
Micronutrients		**uM**
KI	0.75	4.50
H_3BO_3	3.00	50.00
$MnSO_4.4H_2O$	10.00	60.00
$ZnSO_4.7H_2O$	2.00	7.00
$NaMoO_4.2H_2O$	0.25	1.00
$CuSO_4.5H_2O$	0.025	0.10
$CoCl_2.6H_2O$	0.025	0.10
Na_2.EDTA	37.30	100.00
$FeSO_4.7H_2O$	27.80	100.00
Vitamins		
Inositol	100.00	
Nicotinic acid	1.00	
Pyridoxine.HCl	1.00	
Thiamine.HCl	10.00	

Appendix III. 1. Analysis of variance of the effect of 2,4-D concentration, leaf lobes size and incubation period on somatic embryogenesis on semi solid medium (see Section 3.3.1.1.1)

Source	df	SS	MS	VR
2,4-D	3	20.1	6.7	11.2**
period	3	132.7	44.2	73.7**
2,4-D x period	9	43.4	4.8	8.0**
size	1	25.6	25.6	42.7**
2,4-D x size	3	0.3	0.1	0.2
period x size	3	10.6	3.5	5.8*
2,4-D x period x size	9	5.9	0.6	
Total	31	238.59		

Key : * Significant, P>0.05

** Significant, P>0.01

Appendix III.2. Analysis of variance of the effect of 2,4-D concentrations and size of leaf lobes on somatic embryogenesis of cassava cultivar CMC 76 and MCol 22 (see Section 3.3.1.1.2).

Source	df	SS	MS	VR
cultivar	1	61.6	61.6	5.0
size	2	17.3	8.6	0.7
2,4-D	1	46.6	46.6	3.8
cultivar x size	2	9.2	4.6	0.4
cultivar x 2,4-D	1	2.5	2.5	0.2
size x 2,4-D	2	13.5	6.8	0.5
cultivar x size x 2,4-D	2	24.6	12.3	
Total	11	175.3		

Appendix III.3. Analysis variance of the effect of the type of explant on somatic embryogenesis with several cassava cultivars (see Section 3.3.1.1.3)

Source	df	SS	MS	VR
meristem	1	0.9	0.9	0.4
cultivar	3	25.9	8.6	3.9
meristem x cultivar	3	6.6	2.2	
Total	7	33.41		

Appendix III.4. Analysis of variance of the effect of picloram concentration and leaf lobes size on somatic embryogenesis of cultivars CMC 40, CMC 76 and MCol 113 (see Section 3.3.1.2.1)

Source	df	SS	MS	VR
cultivar	2	236.0	118.0	48.2**
size	1	2.4	2.4	0.9
cultivar x size	2	32.3	16.2	6.6*
picloram	4	49.1	12.3	5.0
cultivar x picloram	8	51.1	6.4	2.6
size x picloram	4	9.8	2.4	0.9
cultivar x size x picloram	8	19.6	2.5	
Total	29	400.7		

Key : * Significant, P>0.05

** Significant, P>0.01

Appendix III.5 Analysis of variance of the effect of picloram concentration, leaf lobes size and incubation period on somatic embryogenesis of cultivar CMC 76 (see Section 3.3.1.2.1)

Source	df	SS	MS	VR
picloram	1	112.8	112.8	8.5
size	1	103.1	103.1	7.8
period	1	57.4	57.4	4.3
picloram x size	1	23.7	23.7	1.8
picloram x period	1	71.7	71.7	5.4
size x period	1	0.6	0.6	0.1
picloram x size x period	1	13.3	13.3	
Total	7	382.7		

Appendix III.6. Analysis of variance of the effect of picloram concentration, leaf lobes size and incubation period somatic embryogenesis of cultivar CMC 40 (see Section 3.3.1.2.1)

Source	df	SS	MS	VR
picloram	1	0.1	0.1	0.5
size	1	1.1	1.1	3.8
period	2	48.0	24.0	82.8*
picloram x size	1	0.3	0.3	1.1
picloram x period	2	6.8	3.4	11.7
size x period	2	1.8	0.9	3.0
picloram x size x period	2	0.6	0.3	
Total	11	58.7		

Key : * Significant, P>0.05

Appendix III.7. Analysis of variance of dicamba concentration, and leaf lobes size on somatic embryogenesis of cultivar CMC 76 and MCol 113 (see Section 3.3.1.2.2)

Source	df	SS	MS	VR
cultivar	1	11.9	11.9	99.1**
size	1	2.4	2.4	20.3*
dicamba	3	35.2	11.8	97.9**
cultivar x size	1	16.9	16.9	140.5**
cultivar x dicamba	3	1.8	0.6	4.9
size x dicamba	3	6.3	2.1	17.5*
cultivar x size x dicamba	3	0.4	0.1	
Total	15	74.8		

Key : * Significant, P>0.05

** Significant, P>0.01

Appendix III.8. Analysis of variance of the effect of nitrate : ammonium ratio on somatic embryogenesis of cassava (see Section 3.3.1.3.1)

Source	df	SS	MS	VR
size	2	45.9	22.9	3.8
ratio	3	72.4	24.1	4.0
size x ratio	6	36.1	6.0	
Total	11	154.3		

Appendix III.9. Analysis of variance of the effect of tryptophan and casein hydrolysate added to both Stage-I and Stage-II medium on the production of somatic embryos (see Section 3.3.1.3.2.1)

Source	df	SS	MS	VR
size	2	17.5	8.8	0.7
amino	6	99.2	16.5	1.3
size x amino	12	150.1	12.5	
Total	20	266.9		

Appendix III.10. Analysis of variance of the effect of tryptophan and casein hydrolysate added only to Stage-II medium on the production of somatic embryos (see Section 3.3.1.3.2.1)

Source	df	SS	MS	VR
size	2	80.7	40.3	3.4
amino	4	65.3	16.3	1.4
size x amino	8	95.3	11.9	
Total	14	241.3		

Appendix III.11. Analysis of variance of the effect the pH of tryptophan and casein hydrolysate on the production of somatic embryos (see Section 3.3.1.3.2.1).

source	df	SS	MS	VR
pH	1	2.1	2.1	0.3
size	2	19.2	9.6	1.3
pH x size	2	5.7	2.9	0.4
amino	3	70.6	23.5	3.2
pH x amino	3	16.2	5.4	0.7
size x amino	6	99.4	16.7	2.2
pH x size x amino	6	44.8	7.5	
total	23	257.9		

Appendix III.12. Analysis variance of the effect of the age of clonal plants on embryogenesis in leaf lobes of cultivar CMC 76 (see Section 3.3.1.6)

Source	df	SS	MS	VR
Age	1	85.7	85.7	20.8
Size	2	38.8	19.4	4.7
Age x size	2	8.2	4.1	
Total	5	132.7		

Appendix III.13. Analysis of variance of the effect of 2,4-D concentration and incubation period on somatic embryogenesis in liquid medium (see Section 3.3.2.1)

Source	df	SS	MS	VR
period	1	6.4	6.4	0.8
2,4-D	2	23.3	11.7	1.4
period x 2,4-D	2	17.0	8.5	
Total	5	46.7		

Appendix III.14. Analysis variance of the effect of the size of somatic embryos, concentration of 2,4-D in both liquid and semi-solid medium on the induction of embryogenesis (see Section 4.3.1.1)

Source	df	SS	MS	VR
medium	1	0.54	0.54	108*
size	1	0.83	0.83	166*
2,4-D	2	0.16	0.08	16
medium x size	1	0.99	0.99	198*
medium x 2,4-D	2	0.07	0.04	7
size x 2,4-D	2	3.25	1.63	326**
medium x size x 2,4-D	2	0.01	0.005	
Total	11	5.85		

Key : * Significant, P>0.05

** Significant, P>0.01

Appendix III.15. Analysis variance of the effect of 2,4-D and zeatin on the fresh weight of cells in suspension cultures (see Section 5.3.1.1)

Source	df	SS	MS	VR
time	3	0.3	0.1	1.2
hormone	3	73.2	24.4	243.9**
time x hormone	9	0.9	0.1	
Total	15	74.4		

Key : ** Significant, P>0.01

Appendix III.16. Analysis of variance of the effect of NAA, 2,4-D and BAP on regeneration and production of secondary embryos from fractionated embryos obtained from suspension cultures (see Section 5.3.1.3)

Source	df	SS	MS	VR
fraction	2	4.6	2.3	1.1
hormone	8	39.8	4.9	2.4
Fraction x hormone	16	33.3	2.1	
Total	26			

Appendix III.17. Analysis the effect of sucrose concentration on the survival of somatic embryos slow-cooled to -30^{o}C or direct-cooled to -30^{o}C prior to immersion in liquid nitrogen (using probit transformation, generalised linear model)

Change	df	deviance	mean deviance	F
DMSO	1	17.2	17.2	6.4*
state	2	38.0	19.0	7.0**
sucrose	1	10.0	10.0	3.7
sucrose x DMSO	1	1.1	1.1	0.4
sucrose x state	2	31.2	15.6	5.8*
Residual	28	75.6	2.7	
Total	35	173.1	4.9	

Key : * Significant, P>0.05

** Significant, P>0.01